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**Impact of diet on vascular and biomarkers of injury,  
role of non invasive vascular assessment in  
quantifying risk and the effect of Metformin in  
addressing risk**

By

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## DEDICATION

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## **DECLARATION**

I declare that the work presented in this thesis is my own and has not previously been submitted at this or any other institution. All work described in this thesis was carried during the period of study for this examination and no work carried out previous to that period is included. All publications relating to the work presented in this thesis are attached at the back of the thesis.

All sources have been specifically acknowledged by means of reference.

## SYNOPSIS

Cardiovascular disease (CVD) continues to be a significant cause of mortality in Western society despite improvement in medicine and clinical management. Both CVD and coronary artery disease (CAD) are caused by a pathological process known as atherosclerosis which occurs due to deposition of lipid rich cholesterol material into the arterial inner lining, the endothelium. The endothelium is a thin mononuclear layer that covers the inner surface of all the blood vessels. It acts as an athero-protective organ which mediates its actions through vasoactive mediators, such as nitric oxide (NO). Endothelium derived nitric oxide is synthesised from the amino acid L-arginine by the endothelial nitric oxide synthase (eNOS). The synthesis of nitric oxide by vascular endothelium is responsible for the vasodilator tone which is essential for the regulation of blood pressure and flow. The eNOS enzyme is constitutive, calcium- and calmodulin-dependent, and releases pico-moles of nitric oxide in response to receptor stimulation. A physiologic vaso-dilator tone mediated by nitric oxide is in part maintained through the physical activation of endothelial cells by stimuli such as pulsatile flow and shear stress. This current thesis, as a first stage, investigated vascular function of the endothelium, following a physiological stress, at the macrovascular level (flow mediated dilatation of brachial artery-FMD) in healthy subjects and other CVD risk groups - type 2 diabetes (T2DM), obesity and impaired glucose tolerance (IGT). Oral glucose and high fat meal were used for inducing physiological stress.

On the application of a physiological glucose stress, the mean FMD significantly and profoundly reduced in all CVD groups revealing the acute effects of post-prandial stress on the endothelium ( $p < 0.05$ ).

This thesis, further evaluated the microvascular function at the level of retina (using a retinal vessel analyser, RVA) in all risk groups concurrent with macrovascular function assessment. Such analysis identified that microvascular function, in contrast to FMD, at 1 and 2 hour post

glucose stress, appeared not to reveal significant reduction in healthy population compared with T2DM;

This thesis also explored the impact of high fat diet on markers of endothelial dysfunction, inflammatory markers including TNF- $\alpha$  and endotoxin; which are known to be increased in conditions of CVD and CAD. Studies determined that endotoxin levels increased over time post a high fat meal. Specifically circulating endotoxin levels remained significantly and exponentially high in T2DM even 4 hours after ingesting high fat diet ( $p<0.05$ ); whilst endotoxin levels in healthy subjects revealed only mild raise over time. This thesis explored the role of high endotoxin levels not only in T2DM subjects but also in obese children to demonstrate its association with subclinical inflammation and CVD risk.

As a lipid meal appeared to affect vascular function which was most pronounced in T2DM subjects, this thesis further assessed the potential influence of medication to protect or reduce vascular damage. As such this thesis investigated whether metformin treatment had protective effect on lipoprotein damage and in turn on endothelial function on the high risk group of T2DM compared with control subjects. Glycation, oxidation and nitration reactions lead to significant damage of the apoB100 of LDL increasing atherogenicity and plasma residence times ( $p<0.01$ ). Metformin appeared to reduce this damage ( $P<0.05$  for all AGE (advanced glycation end) products and methionine sulfoxide) and thereby protect against endothelial dysfunction.

This thesis determined the relevance of post-prandial stressors on vascular function at different levels. It investigated the impact such stress could exert on different CVD risk groups and how medication such as metformin may have protective effects. Microvascular reactivity measurements using Retinal vascular analyser appears to demonstrate potential to reliably study acute changes in endothelial function with potential long-term benefits for providing individualized medicine to “at risk” patients.

## **ABBREVIATIONS**

AGE	Advanced glycation end products
AMI	Acute myocardial infarction
apoB100	Apolipoprotein B100
Arg	Arginine
BHF	British Heart Foundation
β cell	Beta cell of pancreas
BMI	Body mass index
BP	Blood pressure
°C	Centigrade
CCD	Charge-coupled device
cGMP	cyclic guanosine 3,5-monophosphate
CAD	Coronary artery disease
CHD	Coronary heart disease
CEL	carboxyethyl-lysine
CO <sub>2</sub>	Carbon-dioxide
CRA	Central retinal artery
CRP	C - Reactive protein
CSRI	Clinical Sciences Research Institute
CHD	Coronary Heart Disease
CMA	N-carboxymethyl-arginine

CML	carboxymethyl-lysine
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DETAPAC	Diethylenetriamine-pentaacetic acid
DNA	Deoxyribonucleic acid
DVA	Dynamic Vessel Analysis
ECG	Electrocardiography
ECHO	Cardiac ultrasound machine
ED	Endothelial dysfunction
EF	Endothelial function
EDHF	endothelium derived hyperpolarising factor
EDRF	endothelium derived Relaxing factor
eNOS	endothelial nitric oxide synthase
EU/ml	Endotoxin Units per milliliter
FFA	Free fatty acid
FL	Fructosyl lysine
FMD	Flow mediated vaso-dilatation
FPG	Fasting plasma glucose
g	Gram
xg	Relative centrifugal force- Gravity
GFR	Glomerular Filtration Rate
G-H1	Glyoxal derivative

GLUT4	Glucose Transporter protein 4
GTP	Guanosine triphosphate
HbA1C	Haemoglobin A1C- Glycosylated Haemoglobin
HDL	High density lipoprotein
Hr	hour
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HSE	Health survey of England
ICAM	Intercellular adhesion molecule
ICAM-1	Intercellular Adhesion Molecule Type-1
IGT	Impaired Glucose Tolerance
IL-6	Interleukin 6
IR	Insulin resistance
kDa	Kilo Daltons
KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate
LC-MS/MS	Liquid chromatography– tandem mass spectrometry
LDL	Low-density lipoprotein
L-arg	L-Arginine
Lys	Lysine
MCP-1	Monocyte Chemoattractant Protein-1
Met	Methionine
MetSO	methionine sulfoxide
MI	Myocardial Infarction

MG-H1	Methylglyoxal derivative
MGmin	Methylglyoxal minimally glycated
Mg/ml	milligrams per milliliter
MHz	Mega Hertz – Unit of frequency
μl	Microliters
ml	millilitres
mol/l	moles per liter
mmol/l	millimoles per liter
MMP-9	Matrix MetalloProteinase-9
MOLD	Methylglyoxal-derived lysine dimer
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NCEP	National Cholesterol Education Program
ND	Non- diabetic subjects
NHS	National Health service, UK
NO	Nitric oxide
NOS	Nitric oxide synthase
O <sub>2</sub>	Oxygen
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate buffered saline
pH	Measure of acidity or alkalinity

PI3K	Phosphatidylinositol-3-OH kinase
PPG	post prandial glucose
RBP4	Retinol binding protein 4
ROS	Reactive oxygen species
RVA	Retinal vessel analyser
RVR	Retinal vessel reactivity
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SCORE	Systematic coronary risk evaluation
sICAM-1	Soluble Intercellular Adhesion Molecule Type-1
sGC	Soluble guanylate cyclase
sVCAM-1	Soluble Vascular Cell Adhesion Molecule-1
TBARS	Thiobarbituric acid reactive substances
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TLR-4	Toll-Like Receptors
TNF- $\alpha$	Tumour Necrosis Factor - $\alpha$
tPA	Tissue Plasminogen Activator
Tyr	Tyrosine
UHCW	University Hospitals Coventry and Warwickshire
UK	United Kingdom



VCAM	Vascular cell adhesion molecule
VEGF	Vascular Endothelial Growth Factor
Wc	Waist circumference
WHO	World Health Organisation
3-DG	3- Deoxy glucosone
3DG-H	Deoxyglucosone derivative
3-NT	3-nitrotyrosine

## **Chapter 1**

### **Introduction**

## **1.1 Cardiovascular disease (CVD)**

### **1.1.1 Prevalence of Cardiovascular Disease**

Cardiovascular disease (CVD) and coronary artery disease (CAD) are caused by a pathological process known as atherosclerosis which occurs due to deposition of lipid rich cholesterol material into the arterial inner lining, the endothelium. Subsequent smooth muscle proliferation and arterial wall thickening result in luminal narrowing, which impacts on blood flow and oxygen delivery to the dependant tissues. At the level of coronary arteries, this process results in symptoms of angina. Further progress of this condition leads to thrombosis when platelets aggregate over the disrupted endothelium. The thrombosis causes further reduction of arterial luminal diameter and acute reduction or complete cessation of blood flow leads to acute myocardial infarction (AMI) at the level of coronary arteries. AMI is a dangerous and life threatening complication of the atherosclerotic process. CAD (Coronary artery disease) accounts for approximately one half of all CVD related deaths (Thom, Haase et al. 2006). CVD continues to be one of the biggest killers in western countries. CAD is the most common type of heart disease, killing over 370,000 people annually in the United States of America. Every year about 735,000 Americans have a heart attack. Of these, 525,000 are a first heart attack and 210,000 happen in people who have already had a heart attack. Data are from the Multiple Cause of Death Files, 1999-2013, as compiled from data provided by the 57 vital statistics jurisdictions through the Vital Statistics Cooperative Program. Underlying Cause of Death 1999-2013 on CDC WONDER Online Database, released 2015 Accessed Feb. 3, 2015. According to the last available Office of National Statistics document “Statistical bulletin: Deaths Registered in England and Wales (Series DR), 2014”, the most common cause of death in males for England and Wales (Rank 1) is Ischemic Heart disease (IHD) (ICD10 code I20-I25). It accounts for 14.8% of all male deaths which is more than twice the

frequency of the Second most common cause of death (dementia and Alzheimer's disease). Similarly for females, IHD continues to be the second most common cause of death in 2014. Age standardized mortality rate per million population for males is 1647 and for females it is 1020 in 2014. Therefore, CVD continues to be a serious medical condition and results in significant burden on the National Health Service (NHS). Similarly, CVD impacts on other serious health conditions like type 2 diabetes mellitus (T2DM) leading to a significant increase in morbidity and mortality. According to the British Heart Foundation (BHF), total CVD burden on the UK is estimated to cost around £30 billion every year. According to the World Health Organization (WHO), the prevalence of CVD in T2DM individuals ranges from 26% to 36%. CVD accounts for as much as 75% of hospital admissions for diabetic complications and 75% of deaths in T2DM subjects (Charbonnel and Del Prato 2003). In view of such a significant impact, urgent measures will be needed, not only to reduce the burden of CVD, but also to prevent it at an earlier stage.

#### 1.1.2 Risks for Cardiovascular Disease (CVD)

T2DM subjects have a two to four fold increased risk of CVD compared with non diabetic, healthy (ND) subjects (Kannel and McGee ; Kannel and McGee 1979; Kannel and McGee 1979; Stamler, Vaccaro et al. 1993). The risk appears to be progressive with raised serum glucose levels. A systematic review found that the relationship between glucose levels and occurrence of cardiovascular events is graded, a relationship that is apparent even for glucose levels below diabetic threshold (Kuusisto, Mykkanen et al. 1994) (Coutinho, Gerstein et al. 1999). Similarly, obese subjects also have a higher risk of developing CVD (Kannel, Gordon et al. 1979). In obesity, prediction of a cardiovascular event risk is not as straight forward as impaired glucose tolerance (IGT) or T2DM because the pathology involved in obesity is

varied. Some of the factors involved include insulin resistance (IR), pro-thrombotic state, pro-inflammatory state and the type of obesity, i.e., android or gynoid, abdominal or otherwise (Rimm, Stampfer et al. 1995; Eckel and Krauss 1998; Grundy 2002). The underlying risk in all of the above groups linking obesity, IGT, T2DM and IR with cardiovascular risk is endothelial dysfunction (Steinberg, Chaker et al. 1996; Caballero 2003; Hsueh, Lyon et al. 2004).

## **1.2 High Risk Groups**

### **1.2.1 Definition of High Risk Groups**

High risk groups are subjects with factors or features which predispose them to high propensity for future CVD. These groups were initially identified by the Framingham heart study in the early 60's (Kannel, Gordon et al. 1979).

### **1.2.2 Coronary Artery Disease (CAD) Risk**

Subsequent to the Framingham heart study, 10 year cardiovascular risk of a healthy individual could be calculated as a percentile risk, referred to as the Framingham risk score. This risk predictive tool was able to identify high risk groups like diabetics, hypertensive's, obese, smokers and subjects with high cholesterol levels (Kannel, Gordon et al. 1979; Kannel and McGee 1979; Kannel and McGee 1979; Kannel and McGee 1979) who each had independent risk of premature CVD compared with healthy controls. However during the subsequent decades post Framingham, it was evident that the risk predictor could be over estimating risk in certain populations who, themselves, had lower baseline risk. Therefore, in

order to develop a risk predictor for Europe, “Systematic coronary risk evaluation” (SCORE) project was developed. This tool can now predict the 10 year risk of fatal cardiovascular events (coronary risk) with different combinations of risk factors (Conroy, Pyorala et al. 2003).

### 1.2.3 Obesity

Obesity is a worldwide health problem which has spread in epidemic proportions throughout most countries. Excess weight gain within populations predicts an increased burden of diseases such as cardiovascular diseases, T2DM and cancers. In a recently published report, using a simulation model, health and economic consequences of a continued rise in obesity over the next two decades in the UK were calculated (Wang, McPherson et al. 2011). These trends projected 11 million more obese adults in the UK by 2030, leading to an additional 544 000–668 000 cases of diabetes, 331 000–461 000 of coronary heart disease and strokes, and 87 000–130 000 of cancer with 2·2–6·3 million quality-adjusted life years lost for UK (Wang, McPherson et al. 2011). The combined medical costs associated with treatment of these preventable diseases were estimated to increase by £1·9–2 billion/year in the UK by 2030 (Wang, McPherson et al. 2011). “Health survey of England (HSE) 2009”, a document published by The NHS National Statistics, identified, obesity or overweight as a significant public health problem in view of its association with significant risk factors for disease and mortality. Body mass index (BMI) and waist circumference are the tools used to identify general obesity and abdominal obesity respectively.

As childhood obesity is increasing in prevalence in the western countries, conditions such as the metabolic syndrome, type 2 diabetes and CHD all tend to have peak prevalence in middle-aged and older individuals but assessments of cardio metabolic risk in childhood and

adolescence become important to define early causal factors and characterise preventive measures. Typically, researchers investigating prospective cohort studies have relied on the thesis that cardiovascular risk factors, such as dyslipidaemia, hypertension and obesity, track from childhood into adult life. A recent review summarises some of the evidence that these factors, when measured in childhood, may be of value in assessing the risk of adult cardio metabolic disease, and as such proceeds to describe some of the methods for assessing cardio metabolic risk in children (Huang, Prescott et al. 2015) (Figure 1.2.3).

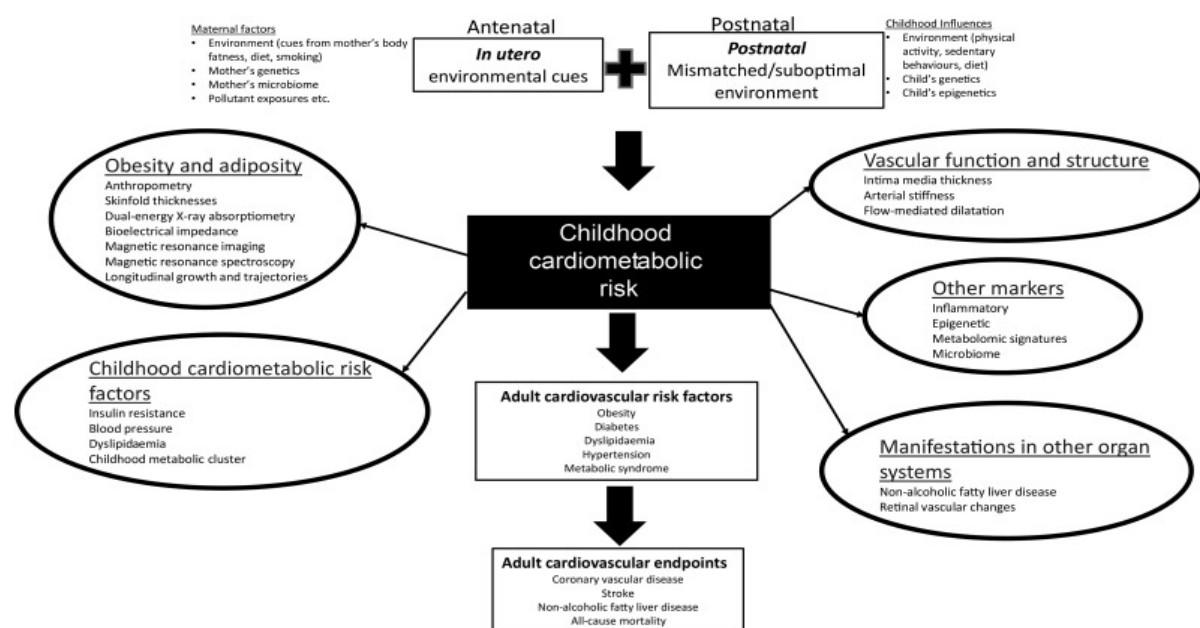


Figure 1.2.3 - Methods for assessing cardio metabolic risk in children (Huang, Prescott et al. 2015).

Obese children are at higher risk of being obese as adults, and adult obesity is associated with an increased risk of morbidity. A systematic review and meta-analysis investigated the ability of childhood body mass index (BMI) to predict obesity-related morbidities in adulthood. It found that high childhood BMI was associated with an increased incidence of adult diabetes

(OR 1.70; 95% CI 1.30-2.22) and coronary artery disease (OR 1.20; 95% CI 1.10-1.31) (Llewellyn, Simmonds et al. 2015).

In 2008, almost a quarter of adults (24% of men and 25% of women aged 16 or over) in England were classified as obese (BMI 30kg/m<sup>2</sup> or over). A greater proportion of men than women (42% compared with 32%) in England were classified as overweight in 2008 (BMI 25 to less than 30kg/m<sup>2</sup>). Thirty-nine per cent of adults had a raised waist circumference in 2008 compared to 23% in 1993. Women were more likely than men (44% and 34% respectively) to have a raised waist circumference (over 88cm for women and 102 cm for men). Using both BMI and waist circumference to assess risk of health problems, for men: 20% were estimated to be at increased risk; 14% at high risk and 21% at very high risk in 2008. Equivalent figures for women were 15% at increased risk; 17% at high risk and 24% at extremely high risk.

Mean BMI increased with age in both sexes up to the age of 74, but dropped back slightly beyond age 75. Almost a quarter of adults (22% of men and 24% of women) were obese. 66% of men and 57% of women were overweight or obese. Among both men and women, prevalence of overweight and obesity was lowest in the 16-24 age groups, and increased in the older groups up to the age of 74. Levels of obesity have increased over the period measured by the Health survey of England (HSE) document 2009. In men, the prevalence of obesity increased from 13% in 1993 to 22% in 2009. In women, 16% were obese in 1993 and 24% were obese in 2009. Recently focus in Europe, and particularly in the UK, has shifted towards the alarming rise in childhood obesity and its subsequent consequences. The annual rate of increase in the prevalence of childhood obesity is currently 10 times higher than it was in the 1970s (WHO Europe 2007 report). Furthermore, as overweight and obesity are responsible for about 80% of cases of T2DM, 35% of ischaemic heart disease and 55% of hypertensive disease among adults in the UK, this new epidemic of childhood obesity (Chinn



and Rona 2001) is extremely concerning all European governments. Obesity and its consequences are already responsible for more than 1 million deaths and 12 million life-years of ill health each year, and taking up to 6% of national health care costs in the WHO European Region (WHO Europe 2007 report). As the burden of illness is predicted to rise exponentially, it is critical that effective preventive strategies are adopted to focus on early identification and risk stratification ultimately to reduce its burden.

#### 1.2.3.1 Definition of Obesity

Obesity is a medical condition where excess body fat accumulation may have an adverse impact on health leading to reduced life expectancy. In adults, excess body weight is defined as having a body mass index (BMI)  $\geq 25$  kg/m<sup>2</sup>. Obesity is defined as a BMI  $\geq 30$  kg/m<sup>2</sup> (World health organization, WHO Europe report 2007). The following table 1.2.3.1 defines the body mass index (BMI) cut off for the definition of overweight and obesity. Even though an attempt was made by the WHO to adopt an alternative BMI chart with more suitable criteria for people of Asian origin, the consensus document currently still holds the following universal BMI cut offs for all definitions of weight parameters (Table 1.2.3.1).

Classification	BMI(kg/m)
Underweight	<18.50
Normal range	18.50 - 24.99
Overweight	$\geq 25.00$
Pre-obese	25.00 - 29.99
Obese	$\geq 30.00$
Obese class I	30.00 - 34.99
Obese class II	35.00 - 39.99
Obese class III	$\geq 40.00$

Table 1.2.3.1 The International Classification of adult underweight, overweight and obesity according to BMI. The World health Organization (WHO) – 2009.

The definition of obesity based solely on BMI measurements meant other vital information might be ignored. Vital information about important factors like age, sex, race, abdominal circumference and waist hip ratio could be missed. These factors have significant influence on risk stratification (Flegal and Ogden 2011). In view of this, the true picture of obesity might not be reflected in the official data collections.

#### 1.2.3.2 Ethnic and Gender Variation

The percentage body fat and BMI vary widely between different ethnic groups, including Caucasians of European and American origin (Deurenberg, Yap et al. 1998; Gurrici, Hartriyanti et al. 1998). This implies that current method of diagnosing obesity solely based BMI/body weight may not be accurate. These studies also reveal that some ethnic groups,

such as Asians, tend to have more body fat for the same BMI compared to Europeans. This means that the true definition of obesity could be different in different ethnic groups (Gurrici, Hartriyanti et al. 1998; Nishida 2004). However, in the absence of any robust alternative, the WHO has continued to use BMI to diagnose obesity across various age, sex and ethnic groups.

Similarly, distribution of body fat is not comparable between the sexes. Men tend to accumulate excess fat around the abdominal region of the body whereas young women appear to predominantly accumulate fat around the hips and thighs. This was originally described in a sentinel paper by Vague in the 1950s (Vague 1956), which distinctly illustrates the Android (male) and Gynoid (female) types of fat distribution in obesity. It also clearly mentions the fact that in every area of the body, except for the first three cervical vertebral region, the fat percentage is higher in women than men (Vague 1956), a finding that translates into differing risk between men and women. Men, with their android distribution, appear to be at a higher risk for diabetes and heart disease compared with women, due to the latter's gynoid distribution of fat. This led to the next stage of obesity measurement, where the differing patterns of fat distribution were taken into account to measure obesity and the concept of waist to hip ratio was formulated (Grundy 2002). Subsequently, various studies have started to look at the best parameters for obesity identification between BMI, waist to hip ratio, waist circumference and weight itself. Studies investigated differences in metabolic effects of obesity (Brook, Bard et al. 2001), (Snijder, Dekker et al. 2003), ultimately trying to identify better predictive tools for measuring risk of diabetes and heart disease (Molarius, Seidell et al. 1999; Molarius, Seidell et al. 1999). A review paper in 1990 demonstrated the significant impact of body fat distribution on the risk of CVD (Després, Moorjani et al. 1990).

### 1.2.3.3 Consequences of Obesity

Obesity is a result of an imbalance between energy expenditure and energy obtained from ingesting food. The resultant excess energy is stored in the fat cells of the body. The consequences of obesity are related to the effects of the increased mass of cells and their metabolic output. The excess fat mass production and distribution in the body is not uniform between subjects, sexes or races, hence creating a differing risk. Apart from physical issues due to the mass of cells and their metabolic reactions, social, behavioural and psychological consequences of obesity are not uncommon (Bray 2004). According to a WHO report, the risk of ill health and other detrimental effects get progressively worse with increasing BMI. The health consequences of obesity can be categorised into CVD, T2DM, insulin resistance, musculo-skeletal disorders – especially osteoarthritis and some types of cancers (e.g. Breast, endometrial and colon). The WHO projects that deaths due to diabetes will increase by more than 50% worldwide in the next 10 years. The heart diseases and stroke are already the world's number one cause of death leading to 17 million deaths every year. Childhood obesity is associated with a higher chance of premature death and disability in adulthood (WHO report 2009). Obesity is considered a global epidemic with important public health issues (Pires, Castela et al. 2015). It is an independent risk factor in the development of cardiovascular disorders. When it is evident in obese children, it potentially contributes towards cardiovascular disease in the adult (Pires, Castela et al. 2015). The anatomic/pathological changes begin early in childhood and studies in children with excess weight or obesity have shown endothelial changes that represent the precursors of the atherosclerotic lesions (Pires, Castela et al. 2015).

The most important of these consequences, T2DM, will be explored further in this chapter. Even though the development of T2DM occurs due to concurrent multi-factorial metabolic

factors, obesity represents the single most important factor for the pathogenesis of T2DM. The epidemiological link between obesity and diabetes is so closely inter-linked that the common syndrome has been termed ‘diabesity’ since early 2000. This epidemiological relationship between obesity and T2DM has been highlighted by numerous studies (Chan, Rimm et al. 1994; Colditz, Willett et al. 1995; Wannamethee and Shaper 1999; Mokdad, Ford et al. 2003), demonstrating the increasing incidence of T2DM with increasing BMI. Obesity results in IR, increasing insulin secretion and ultimately metabolic syndrome (Bray 2004). A recent review provides good mechanistic insights into obesity and its consequences including cardio metabolic syndrome (Lovren, Teoh et al. 2015).

#### 1.2.3.4 Obesity and Insulin Resistance (IR)

IR appears to be the primary mechanism by which obesity leads to development of metabolic and vascular complications. These complications include T2DM, dyslipidaemia, hypertension and CVD. IR is a condition where normal amounts of insulin are inadequate to produce an expected insulin response from fat (adipose), muscle and liver cells. The exact mechanisms leading to the development of IR in obese individuals are unclear. Various theories and mechanisms have been put forward over the past few decades to explain the link between obesity and IR. As the interplay between insulin and glucose is most significant in skeletal muscle, adipose tissue (fat) and liver, most of the research has concentrated on these tissues, and continues to do so, in order to understand the underlying causes of IR.

IR can also be seen in normal physiologic conditions during puberty, pregnancy and old age (Kahn, Hull et al. 2006). Similarly, increased physical activity reduces IR (Chen, Bergman et al. 1988; Kahn, Hull et al. 2006). Adipose tissue, being a metabolically active endocrine tissue, normally releases various hormones such as adiponectin, some pro-inflammatory

cytokines, glycerol and free fatty acids (FFA). Most of these products are produced in excess in obesity, which is important in the progression of IR, particularly as FFA have the potential to induce IR and impair beta cell function (Roden, Price et al. 1996; Kahn, Hull et al. 2006). Routinely, insulin would inhibit the release of FFA from adipose tissue (Rabinowitz and Zierler 1962). However, if plasma insulin levels are not able to rise with increasing FFA production and release, then circulating FFA levels will increase. This will eventually lead to elevated blood glucose levels by increasing hepatic glucose production and hyperglycaemia (Reaven 1988). FFA induces insulin resistance by inhibition of glucose phosphorylation and oxidation, ultimately resulting in reduced muscle glycogen synthesis and hyperglycaemia (Roden, Price et al. 1996). In obese subjects, FFA ultimately fails to stimulate insulin secretion (Boden 1997). Another critical product which induces IR in obese subjects is retinol binding protein 4 (RBP4) (Yang, Graham et al. 2005; Kahn, Hull et al. 2006). RBP4 is the only specific transport protein for delivering retinol to tissues. Liver and adipose cells are the main source of circulating RBP4. As the adipose cell mass increases, RBP4 production from these cells also increases. At the same time, RBP4 clearance from plasma is also altered in obese subjects, explaining the observed increase in RBP4 levels (Yang, Graham et al. 2005). RBP4 leads to insulin resistance by increasing hepatic glucose output and impairing insulin signalling in muscle (Yang, Graham et al. 2005). Furthermore, it can reduce phosphatidyl inositol-3-OH kinase (PI3K) signalling in muscle and enhance gluconeogenesis in the liver. (Kahn, Hull et al. 2006).

In obese subjects, alteration in glucose transportation systems also leads to insulin resistance. This system consists of a group of 5 homologous transmembrane proteins which are involved in facilitated transport of glucose down the concentration gradient. Glucose uptake is the rate-limiting step in glucose utilization in mammals and is tightly regulated by a family of specialized proteins, called the facilitated glucose transporters (GLUTs). There are two types

of glucose transporters. One of them is the sodium dependent glucose transporters (SGLTs) which transport glucose against its concentration gradient and the other- sodium independent glucose transporters (GLUTs), which transport glucose by facilitative diffusion in its concentration gradient (Jurcovicova 2014). The GLUT1 is produced in brain microvasculature and ensures glucose transport across the blood brain barrier (BBB). GLUT2 is present in hypothalamic neurons and serves as a glucose sensor in regulation of food intake. GLUT3 is the most abundant glucose transporter in the brain having five times higher transport capacity than GLUT1. It is present in neurones, mostly in axons and dendrites. Its density and distribution correlate well with the local cerebral glucose demands (Jurcovicova 2014). GLUT5 is predominantly fructose transporter. GLUT4 and GLUT8 are insulin-regulated glucose transporters (Jurcovicova 2014). GLUT4, the major isoform in insulin-responsive tissue, translocates from an intracellular pool to the cell surface and determines insulin-stimulated glucose uptake (Lacombe 2014). In muscle and adipose cells, the predominant glucose transporter isoform is GLUT4. Studies have observed that insulin resistant adipocytes from obese subjects show depletion of GLUT4 transporters (Shepherd and Kahn 1999), with as much as 40 to 80 % depletion of GLUT4 in adipocytes from these subjects (Garvey, Maianu et al. 1991). Similarly in skeletal muscle, the principal site of glucose disposal, insulin resistant cells show decreased glucose transport activity (Lockwood and Amatruda 1983; Friedman, Dohm et al. 1992). However, these effects are not necessarily irreversible, as weight loss has been shown to improve glucose transport activity (Friedman, Dohm et al. 1992). The adverse effects on glucose transport in the adipocyte and skeletal muscle cell may be further compounded by the negative effects of obesity on the beta ( $\beta$ ) cell of the pancreas. The  $\beta$  cell is crucial in ensuring that plasma glucose concentrations are maintained within a narrow physiological range but, in obesity,  $\beta$  cell function and its insulin sensitivity are reduced leading to IR (Kahn, Hull et al. 2006). In the early stages of IR, the

effects can be counteracted by an increase in fasting insulin levels, balancing hepatic glucose production in response to glycaemic load. At a later stage, increases in insulin secretion will be unable to overcome the effect of whole body insulin resistance, at which point the condition progresses to a state of impaired glucose tolerance (IGT).

#### 1.2.4 Impaired Glucose Tolerance (IGT)

IGT is a condition in which fasting plasma glucose levels are higher than normal but lower than those diagnostic of diabetes mellitus. In some subjects, this can progress towards diabetes but, in others, this condition could stabilise or even regress. The diagnosis of IGT is currently based on the 2006 WHO criteria, with fasting glucose levels of  $\leq 7.0$  mmol/l and 2 hour post plasma glucose load of  $\geq 7.8$  mmol/l and  $< 11.1$  mmol/l. IGT usually progresses towards T2DM. Insulin resistance is a common finding in the majority of IGT subjects (Reaven 1988), obese children and adolescents (Invitti, Guzzaloni et al. 2003). The onset of IGT in all these subjects is associated with development of hyperinsulinaemia (Polonsky, Sturis et al. 1996). Hyperinsulinaemia is a state of increasing insulin levels over time. This leads to decreasing insulin sensitivity and impaired pancreatic  $\beta$  cell function, which will ultimately lead to T2DM (Lillioja and Bogardus 1988).

#### 1.2.5 Type 2 Diabetes Mellitus (T2DM)

Several follow up studies have looked at predictive markers of increased T2DM risk in obese and IGT subjects. Further progression to T2DM depends, not only on IR, but sometimes on the gradual failure of the pancreas to maintain high levels of insulin production (Bogardus, Lillioja et al. 1984; Saad, Knowler et al. 1989; Polonsky, Sturis et al. 1996). As insulin



production drops, the subjects progress from IGT to overt diabetes. One of the most powerful indicators for predicting the onset of diabetes is initial blood glucose levels (O'Sullivan and Mahan 1965; Jarrett, Keen et al. 1979; Keen, Jarrett et al. 1982; Sasaki, Suzuki et al. 1982; Kadowaki, Miyake et al. 1984). Multivariate analysis revealed diminished insulin response and high level of fasting and 2-hr glucose values at the initial 100-g oral glucose tolerance test as significant predictive independent risk factors for the development of diabetes in Japanese subjects (Kadowaki, Miyake et al. 1984). In other studies, it has been estimated that up to 70 % of IGT subjects eventually develop diabetes (Jarrett, Keen et al. 1979; Sasaki, Suzuki et al. 1982; Shaw, Zimmet et al. 1999; Gabir, Hanson et al. 2000; de Vegt, Dekker et al. 2001; Nathan, Davidson et al. 2007). However, the natural history of IGT subjects is variable. It appears that around 25% of IGT subjects, over a period of 3 to 5 years, progress to T2DM, whilst another 25% might regress back to ND, but the majority may remain in the same altered glucose state (Nathan, Davidson et al. 2007). Finally, as this state is considered pre-diabetic, it is still possible to reverse this condition both by physical activity and by medication (Tuomilehto, Lindström et al. 2001; Knowler, Barrett-Connor et al. 2002). Even though the 2 hour oral glucose tolerance test (OGTT) is regarded as the gold standard for identification of these groups, it is not used as widely as required. Recent studies have shown that prediction models can also estimate the risk of developing T2DM at the pre-diabetes stage with similar confidence (Stern, Williams et al. 2002). Although there are practical benefits in using a predictive model, it is not, at present, a widely used method, even though obese and IGT subjects are important to evaluate, in view of their high risk of developing CVD.

Once T2DM sets in, life expectancy is predicted to be 5 to 10 years lower than in the ND general population (Charbonnel and Del Prato 2003). The relationship between diabetes and cardiovascular events is so stringent that the American Diabetes Association has identified

diabetes as a type of CHD itself. The National Cholesterol Education Program Expert Panel (NCEP) on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) agree with the conclusion that diabetes should be considered as a CHD risk equivalent (Charbonnel and Del Prato 2003). In the same vein, it is now highlighted that hyperglycaemia, in addition to hypertension and dyslipidaemia, should also be considered an independent risk factor for CVD in diabetic subjects (Ceriello 2005; Ceriello 2009).

Hyperglycemia continues to be an important threat to the health of patients, independent of diabetes status. A recent retrospective cohort study comparing 1- and 5-year risk of death and cardiovascular (CV) complications in patients with a diagnosis of diabetes versus those without diabetes but found to have hyperglycemia at hospital admission for risk of all-cause death and CV complications (acute myocardial infarction [AMI], revealed the following. Compared to patients with diagnosed diabetes, patients with peak serum glucose level >200 mg/dL had significantly higher 1-year risk (hazard ratio [HR]: 1.31, 95% confidence interval [CI]: 1.20-1.43) and 5-year risk (HR: 1.13, 95% CI: 1.06-1.22) of death. In-hospital hyperglycemia is an important clinical indicator, carrying a higher 1- and 5-year mortality risk than those with diagnosed diabetes (Tuna, Manuel et al. 2014).

### **1.3 Post-prandial Stress**

The post prandial state is characterised by increasing levels of both glucose and triglycerides, with the time to peak measurement for both glucose and triglyceride levels varying in different subjects. Similarly the value of peak measurements differs between healthy individuals and diabetic subjects. The post prandial phase, characterised by both hyperglycaemia and hypertriglyceridaemia, may lead to endothelial dysfunction by producing

oxidative stress (Ceriello, Taboga et al. 2002), a phenomenon that is often represented as post-prandial stress.

### 1.3.1 Post-prandial Glucose

Obesity, IGT and T2DM reveal endothelial dysfunction at an early stage (Lam and LeRoith 2000). Post-prandial glucose (PPG) can be used as a common denominator and stressor for all the above conditions to differentiate and evaluate the endothelial function between the above groups. Hyperglycaemia induces endothelial dysfunction by producing oxidative stress (Kawano, Motoyama et al. 1999; Marfella, Quagliaro et al. 2001; Ceriello, Taboga et al. 2002). As such, PPG has already been used in previous studies as a physiological stress inducer to evaluate endothelial function (Ceriello, Taboga et al. 2002; Ceriello, Quagliaro et al. 2004). PPG can lead to the production of Advanced Glycation End products (AGE) (Wolff and Dean 1987) which can lead to production of oxidative stress. AGEs are created by a non-enzymatic reaction between reducing sugars and biological proteins. These compounds accumulate slowly throughout the life span and contribute to structural and physiological changes that can lead to endothelial dysfunction and subsequent CVD (Zieman and Kass 2004). PPG has thus been suggested as an independent risk factor for CVD (Ceriello 1998) in T2DM subjects. Similarly, PPG may also be considered an independent risk factor for the development of retinopathy in diabetes and in IGT (Shiraiwa, Kaneto et al. 2005). Recent evidence suggests that post-challenge hyperglycaemia is a better predictor of cardiovascular risk than fasting glucose. Acute glucose elevations have been associated with a reduced endothelial-dependent flow mediated dilation indicating a decrease in nitric oxide production (Siervo, Corander et al. 2011). Post-prandial hyperglycaemic peaks have been directly associated with increased intima media thickness in type 2 diabetic patients indicative of an

increased atherosclerotic risk (Siervo, Corander et al. 2011). The increase in intra-cellular glucose concentrations in the endothelial cells induces a hyper-generation of reactive oxygen species via the activation of different pathways (polyol-sorbitol, hexosamine, advanced glycated end products, activation of PKC, asymmetric dimethylarginine (ADMA)). These mechanisms influence the expression of genes and release of signalling and structural molecules involved in several functions (inflammation, angiogenesis, coagulation, vascular tone and permeability, cellular migration, nutrient metabolism). ADMA is considered as a biomarker of endothelial dysfunction and it has been associated with an increased risk of atherosclerosis and cardiovascular diseases. The increased generation of ADMA and reactive oxygen species in subjects with persistent hyperglycaemia could lead to an impairment of nitric oxide synthesis (Siervo, Corander et al. 2011).

### 1.3.2 Post-prandial Lipids

Post-prandial hypertriglyceridaemia seems to exert a negative influence on endothelial function, an effect that has been reported in both normal and T2DM subjects (Anderson, Evans et al. 2001; Bae, Bassenge et al. 2001; Ceriello, Taboga et al. 2002). This negative effect seems to be mediated through the production of oxidative stress, resulting in nitric oxide (NO) inactivation (Ceriello, Taboga et al. 2002). The change in postprandial triglycerides is also relevant to CVD (Nordestgaard, Benn et al. 2007), as postprandial hypertriglyceridaemia and dyslipidaemia are well-recognised risk factors for CVD in T2DM (Taskinen, Lahdenpera et al. 1996; Taskinen 2003; Pastromas, Terzi et al. 2008) (Karpe, Steiner et al. 1994; Ceriello 2006; Ceriello, Davidson et al. 2006). In non-obese, T2DM patients with moderate fasting hypertriglyceridaemia, the atherogenic lipoprotein profile are amplified in the postprandial state (Cavallero, Dachet et al. 1994). This raises the possibility

that, in patients with postprandial hyperglycaemia and a concomitant increase in postprandial hyperlipidaemia, the latter might be the true risk factor (Heine and Dekker 2002).

### 1.3.3 Mechanism of Post-prandial Stress

The post-prandial state, as evidenced by acute hyperglycaemia, leads to an increase in nitrotyrosine and peroxynitrite levels. The rise in peroxynitrite levels instigates cytotoxic reactions in the cells, including lipid peroxidation and protein oxidation. This process also involves excess generation of superoxide, which inactivates NO leading to endothelial dysfunction (Ceriello, Taboga et al. 2002). This mechanism of post-prandial hyperglycaemia and the subsequent endothelial dysfunction appears to occur both in healthy subjects (Marfella, Quagliaro et al. 2001) as well as subjects with IGT or T2DM (Marfella, Nappo et al. 2000). Similarly, hypertriglyceridaemia, as part of the post-prandial state, produces a similar response to post-prandial hyperglycaemia, promoting endothelial dysfunction in both healthy and T2DM subjects through peroxynitrite formation (Anderson, Evans et al. 2001).

## 1.4 Assessment of Risk

### 1.4.1 Vascular Endothelium

The endothelium is a thin mononuclear layer that covers the inner surface of all the blood vessels. It separates the circulating blood from the vascular smooth muscle and tissues. The healthy vascular endothelium functions as an athero-protective organ which mediates its actions through vasoactive mediators, such as NO, prostacyclin, and endothelium derived hyperpolarising factor (EDHF).

### 1.4.2 Anatomy of Endothelial Cell

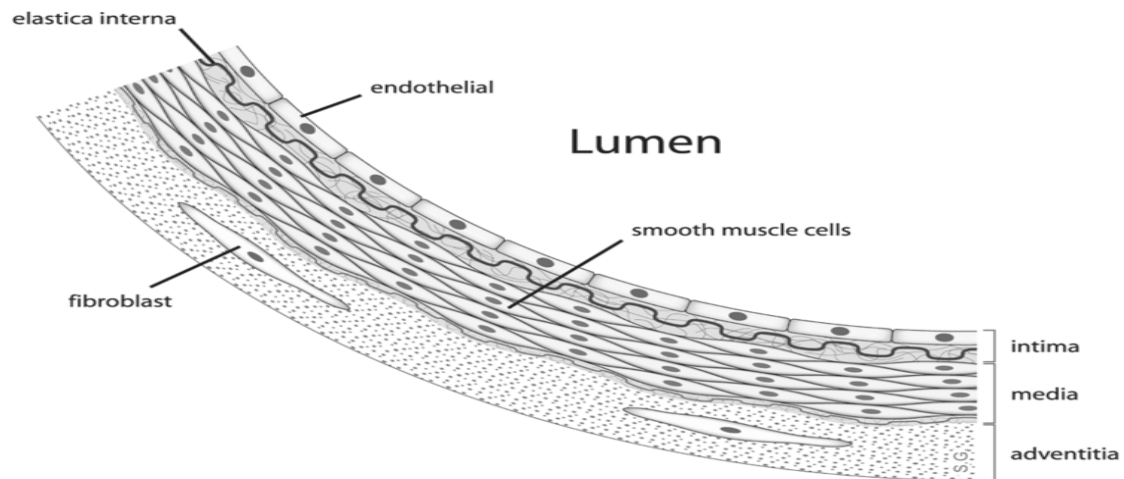


Figure 1.4.2 Endothelial cell: It is a three layered cell structure with an inner lining, intima, smooth muscle laden media and an outer adventitious coat.

### 1.4.3 Function of Endothelium

The endothelium influences vasomotion, platelet and leukocyte adhesion and plaque stabilisation. It senses mechanical stimuli, such as pressure and sheer stress, and hormonal stimuli, such as vasoactive substances. In response to such stimuli, it releases agents that regulate vasomotor function, trigger inflammatory processes and affect haemostasis. It also contributes to the control of vascular tone and local blood flow (Marsden, Goligorsky et al. 1991). The healthy endothelium mediates coagulation, immune function, mitogenesis, angiogenesis, vascular permeability and fluid balance and plays a central role in cardiovascular control (Poredos 2001; Poredos 2002; Landmesser, Hornig et al. 2004).

#### 1.4.4 Mechanism of Nitric oxide release

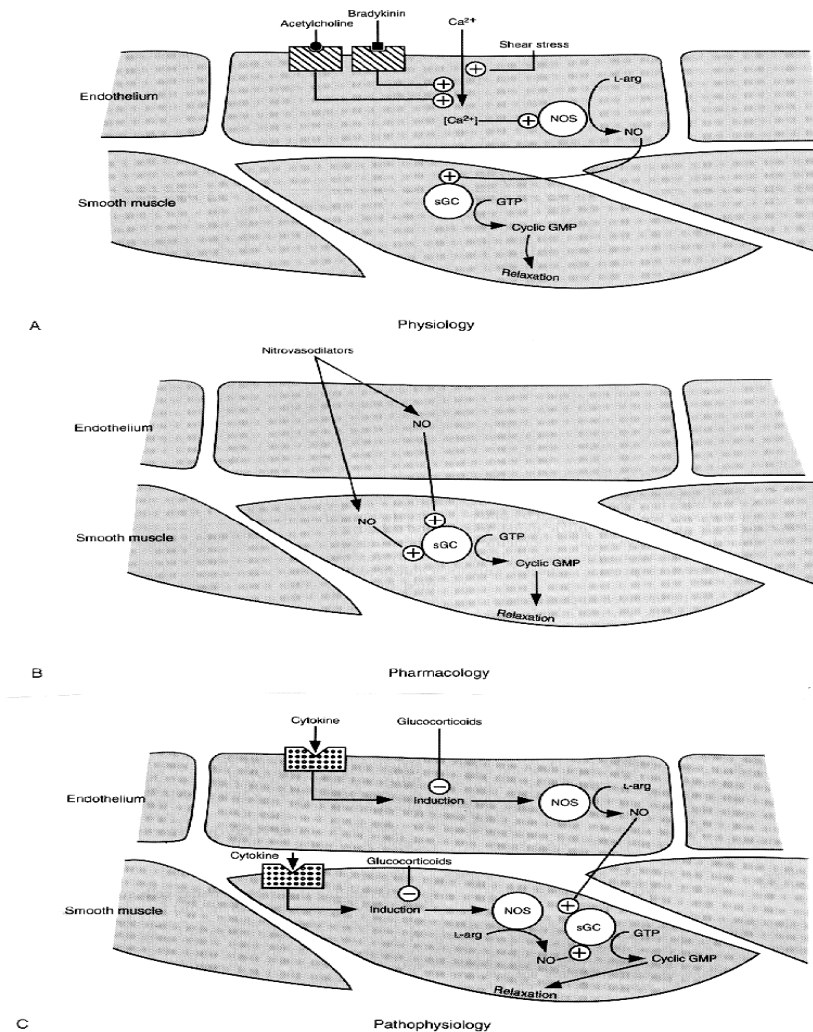


Figure 1.4.4 Vascular effects mediated by Nitric Oxide (Moncada and Higgs 1993).

In panel A, shear stress or receptor activation of vascular endothelium by bradykinin or acetylcholine results in an influx of calcium. The consequent increase in intracellular calcium stimulates the constitutive nitric oxide synthase (NOS). The nitric oxide (NO) formed from L-arginine (L-arg) by this enzyme diffuses to nearby smooth-muscle cells, in which it stimulates the soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP from guanosine triphosphate (GTP). This increase in cyclic guanosine monophosphate (cGMP) in the smooth-muscle cells leads to their relaxation. In Panel B, vasodilators such as sodium nitroprusside and nitroglycerin release nitric oxide, spontaneously or through an enzymatic

reaction. The liberated nitric oxide stimulates the soluble guanylate cyclase in the vascular smooth-muscle cell, resulting in relaxation. In Panel C, the interaction of cytokines with their receptors on endothelial and smooth-muscle cells results in the induction of calcium-independent nitric oxide synthase. This induction is inhibited by glucocorticoids. Once induced, the nitric oxide synthase produces nitric oxide continuously, resulting in sustained activation of soluble guanylate cyclase.

Nitric oxide from the vascular endothelium is released in different ways. There is a continuous basal synthesis of nitric oxide which is released to maintain resting vascular tone (Vallance and Chan 2001). Secondly, both chemical mediators and acetylcholine apart from physical stimuli like shear stress can activate endothelial nitric oxide synthase (eNOS) and lead to increased nitric oxide production and release (Vallance and Chan 2001). All the mechanisms involved in activation and release of nitric oxide are depicted pictographically above (Vallance and Chan 2001) in Figure 1.4.4.

Endothelium derived nitric oxide is synthesised from the amino acid L-arginine by the endothelial nitric oxide synthase (eNOS). The synthesis of nitric oxide by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure. The eNOS enzyme is constitutive, calcium- and calmodulin-dependent, and releases pico-moles of nitric oxide in response to receptor stimulation (Moncada 1992; Moncada and Higgs 1993). A physiologic vaso-dilator tone mediated by nitric oxide is essential for the regulation of blood flow and pressure. This nitric oxide-dependent vasodilator tone seems to be maintained through the physical activation of endothelial cells by stimuli such as pulsatile



flow and shear stress (Moncada 1992; Moncada and Higgs 1993). Nitric oxide is a labile gas with a very short half life of  $< 4$  seconds in biological solutions (Vallance and Chan 2001).

It is rapidly oxidised to nitrite before being excreted into the urine (Moncada and Higgs 1993; Vallance and Chan 2001). Once synthesised, the nitric oxide rapidly diffuses across the endothelial cell membrane and enters the vascular smooth muscle cells where it activates guanylate cyclase, leading to an increase in intracellular cyclic guanosine-3',5-monophosphate (cGMP). cGMP, acting as a second messenger, mediates many of the biological effects of nitric oxide including the control of vascular tone and platelet function (Vallance and Chan 2001).  $\text{Ca}^{2+}$ -activated calmodulin is important for the regulation of eNOS activity. Endothelial NOS synthesizes NO in a pulsatile manner with eNOS activity markedly increasing when intracellular  $\text{Ca}^{2+}$  rises.  $\text{Ca}^{2+}$  induces the binding of calmodulin to the enzyme (Hemmings and Mayer 1998; Forstermann and Sessa 2012). eNOS can also be activated by stimuli that do not produce sustained increases in intracellular  $\text{Ca}^{2+}$ , but still induce a long-lasting release of NO. The best established such stimulus is fluid shear stress. This activation is mediated by phosphorylation of the enzyme (Fulton, Gratton et al. 1999; Fleming and Busse 2003; Forstermann and Sessa 2012). The eNOS protein can be phosphorylated on several serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. Phosphorylation of Ser1177 stimulates the flux of electrons within the reductase domain, increases the  $\text{Ca}^{2+}$  sensitivity of the enzyme, and represents an additional and independent mechanism of eNOS activation (Fleming and Busse 2003; Forstermann and Sessa 2012). Even though all the kinases above can regulate eNOS Ser1177 in vitro, Akt1(Ser/Thr kinase) is the only kinase proven to regulate eNOS function in vivo (Forstermann and Sessa 2012).

#### 1.4.5 Mechanism of oxidative stress

Oxidative stress in chemical terms is a large increase in the cellular reduction potential. It is caused by an imbalance between the production of reactive oxygen and the biological system's ability to quickly detoxify. The reducing environment in all mammalian cells is preserved by constant input of energy by the enzymes. Any imbalance in the normal redox state including hyperglycemia (Selvaraju, Joshi et al. 2012) could lead to toxic effects on the cell by production of free radicals and peroxides. Most destructive aspect of oxidative stress is the production of reactive oxygen species which include peroxides and free radicals. Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells. Excessive production of ROS, outstripping endogenous antioxidant defence mechanisms, has been implicated in processes in which they oxidize biological macromolecules, such as DNA, protein, carbohydrates, and lipids. This condition has commonly been referred to as oxidant stress (Cai and Harrison 2000). Of all the proteins, LDL oxidation is central to the development of atherosclerosis and associated endothelial dysfunction (Anderson 2003). Similarly oxygen free radicals also lead to endothelial dysfunction (Chen, Wen et al. 2015) and risk of CVD (Selvaraju, Joshi et al. 2012) by directly inactivating NO (Rubanyi and Vanhoutte 1986; Anderson 2003). Oxidant stress is now shown to be involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure (Cai and Harrison 2000). Superoxide anion interacts with nitric oxide forming the potent toxin peroxynitrite via diffusion limited reaction, which in concert with other oxidants triggers activation of stress kinases, endoplasmic reticulum stress, mitochondrial and poly (ADP-ribose) polymerase 1-dependent cell death, dysregulates autophagy/mitophagy, inactivates key proteins involved in myocardial calcium handling/contractility and antioxidant defense, activates matrix metalloproteinases and redox-dependent pro-inflammatory transcription

factors (e.g. nuclear factor kappaB) promoting inflammation, AGEs formation, eventually culminating in myocardial dysfunction, remodelling and heart failure (Varga, Giricz et al. 2015).

#### 1.4.6 Endothelial Dysfunction (ED)

Endothelial dysfunction is a physiological dysfunction of normal biochemical processes carried out by the endothelium. It is thought to be a key event in the development of atherosclerosis (Landmesser, Hornig et al. 2004) and predates clinically obvious vascular pathology by many years (Poredos 2002). Endothelial dysfunction has also been shown to be of prognostic significance in predicting vascular events (Vita and Keaney 2002; Quyyumi 2003). A systematic review by Garcia in 2012 clearly demonstrated that brachial artery flow mediated dilatation measuring endothelial dysfunction predicts cardiovascular risk (Garcia, Lima et al. 2012). A key feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to an appropriate stimulus. Dysfunctional endothelial cells are unable to produce NO to the same extent (or there is increased and rapid destruction of NO) as healthy endothelial cells, and therefore vasodilatation, is reduced (Candido and Zanetti 2005). This creates a detectable difference in subjects with endothelial dysfunction compared to a normal, healthy endothelium.

#### 1.4.7 Inflammation and Endothelial Dysfunction

Obesity and T2DM are associated with sub-clinical inflammation, which is related to adipose tissue nutrient metabolism (Schoeller and Buchholz 2005; Bakker, Eringa et al. 2009). Pro-inflammatory state in adipocytes is created in obese subjects because of nutrient storage

rather than steady-state nutrient and glucose concentrations (Bakker, Eringa et al. 2009). This pro-inflammatory state and subsequent release of inflammatory markers lead to Insulin resistance, thrombogenic risk and immune response (Roden, Price et al. 1996; Boden 1997; Kahn and Flier 2000; Grundy 2002; Kahn, Hull et al. 2006; Bakker, Eringa et al. 2009). The common factors expressed include TNF- $\alpha$ , IL-6, PAI-1, resistin and leptin. Endothelial injury results in the release of various factors that can be detected in the circulation, and these can then be used as markers of endothelial dysfunction. Circulatory markers of endothelial dysfunction most often used are: endothelin-1, von Willebrand factor, tissue Plasminogen Activator (tPA) and Plasminogen Activator Inhibitor-1 (PAI-1) and the adhesion molecules, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), P-selectin, as well as amyloid A, fibrinogen, Oxidised LDL-C, homocysteine, C-Reactive protein (CRP), visfatin, adiponectin and AGE products (Gimbrone 1999; Jager, van Hinsbergh et al. 2000; Bakker, Eringa et al. 2009).

#### 1.4.8 Triggers for Endothelial Dysfunction

Endothelial dysfunction is, in part, a consequence of the harmful effects of the risk factors of atherosclerosis on the vessel wall. Atherosclerosis is a chronic pathological process of hardening and loss of elasticity of arteries. This process starts as early as childhood and can accelerate with the addition of traditional identifiable risk factors for CAD. It is asymptomatic in the early stages but, over a period of decades, can promote plaque formation which may ultimately result in plaque rupture and vascular events, including myocardial infarction (MI). One tool which is widely used to predict CHD risk is the 10 year Framingham risk score with its limitations. It is mainly based on traditional and conventional cardiac risk factors. But, it is also recognised that a sizable chunk of the population, classed as low risk on the Framingham

risk model, in fact have sub-clinical atherosclerosis, which this tool cannot detect or predict. Other models include NEW-CHD model (Hu, Root et al. 2014) and multilocus genetic risk score (MGRS) models (Ganna, Magnusson et al. 2013). Risk factors for atherosclerosis include raised blood pressure (hypertension), increased cholesterol concentration (hypercholesterolaemia), raised glucose levels / hyperglycaemia (diabetes), elevated homocysteine levels, increased pro-inflammatory markers, and constituents of cigarette smoke – all of which are known to diminish endothelial function (Vallance and Chan 2001; Poredos 2002). Endothelial dysfunction is associated with impaired tissue perfusion, particularly during stress, which might contribute to ischemia including myocardial ischemia (Drexler and Hornig 1999). Subjects with hypertension, T2DM, hypercholesterolaemia and people who smoke have all been shown to have endothelial dysfunction, thus predicting future CVD (Chowienczyk, Watts et al. 1992; Sorensen, Celermajer et al. 1994; Heitzer, Yla-Herttuala et al. 1996; Poredos 2002). Several mechanisms may be involved in the development of endothelial dysfunction, such as reduced synthesis and release of EDRF (Drexler and Hornig 1999), reduced nitric oxide synthase (NOS) production (Sukhovshin, Yepuri et al. 2015), over production of endogenous inhibitors of NOS, oxidative stress, reduced sensitivity of vascular smooth muscle and production of AGE (Vallance and Chan 2001). Increased plasma levels of oxidized LDL have been noted in chronic smokers and are related to the extent of endothelial dysfunction, raising the possibility that chronic smoking potentiates endothelial dysfunction by increasing circulating and tissue levels of oxidized LDL (Drexler and Hornig 1999). Currently various modalities are used to limit and reduce the cardiovascular events.

Endothelial dysfunction is an early CV risk marker in obese men and women with prediabetes. Overweight and obese status denotes an increasing adipose tissue burden which spills over into ectopic locations, including the visceral compartment, muscle and liver.

Associated co-morbidities enhance cardiovascular (CV) risk. (Gupta, Ravussin et al. 2012). Endothelial dysfunction (ED) is the initial perturbation, which precedes fatty streak known to initiate atherosclerosis: insidious process which often culminates as sudden catastrophic CV adverse event. Endothelial dysfunction reflective of cardio metabolic changes in obese adults can be an early risk marker for catastrophic CV events (Gupta, Ravussin et al. 2012).

Invasive modalities like coronary angiograms are relied upon to rule out existing CAD but there are limitations in interpreting their findings. The most important one, apart from being a two dimensional technique, is that it can only observe changes seen in the lumen of the vessel. The atherosclerotic process starts within the wall of the artery. Early changes of atherosclerosis are unlikely to be appreciated by a diagnostic angiographic procedure. Non-invasive vascular tests can detect some of the vascular wall changes, mainly functional changes, thus enabling detection of early stages of the atherosclerosis.

#### 1.4.9 Assessment of Endothelial Dysfunction

Endothelial dysfunction can be assessed both at the level of coronary arteries as well as other accessible medium size vessels which correlate with coronary endothelial dysfunction and coronary events. Whilst assessing endothelial dysfunction in coronary arteries involves invasive equipment and studies, (Bossaller, Hehlert-Friedrich et al. 1989; Chu, Chambers et al. 1991; Fujino, Mikuniya et al. 1993; van Haare, Kooi et al. 2015), similar assessment in other accessible medium vessels can be carried out non-invasively. Since, endothelial dysfunction has been proven as an early event of patho-physiological importance in the atherosclerotic process, (Celermajer, Sorensen et al. 1992) there is continued interest in the methods used to assess such risk, at an early stage.

Endothelial dysfunction has also been shown to provide an important link between diseases such as obesity (Steinberg, Chaker et al. 1996), hypertension, chronic renal failure, angina or diabetes (Hsueh, Lyon et al. 2004) and the high risk for cardiovascular events (Suwaidi, Hamasaki et al. 2000). This means that a large section of the high risk population could potentially be identified if a reliable and less invasive system could be developed. As such, this led to the development of non-invasive scanning methods, which studied endothelial function at accessible vessels such as carotid artery and brachial artery. Determination of endothelial dysfunction also has important clinical implications. It was demonstrated that endothelial dysfunction, at the coronary artery level, is significantly and independently correlated with the occurrence of cardiac events including cardiovascular death, unstable angina, myocardial infarction, percutaneous transluminal coronary angioplasty, coronary bypass grafting, ischemic stroke, or peripheral artery revascularization (Schächinger, Britten et al. 2000).

Similarly, that endothelial dysfunction, at the level of peripheral vasculature, also found evidence of prognostic value. Therefore, non-invasive endothelial function testing of forearm vessels predicted cardiovascular events in patients with CAD (Heitzer, Schlinzig et al. 2001), peripheral artery disease (Gokce, Keaney et al. 2003) and hypertension (Perticone, Ceravolo et al. 2001). In addition, non-invasive testing using ankle –arm index (Newman, Shemanski et al. 1999), common carotid artery (Bots, Hoes et al. 1997) and femoral artery (Belcaro, Nicolaidis et al. 2001) scanning have also shown evidence towards predicting future cardiac events. Brachial artery FMD predicts cardiovascular risk (Garcia, Lima et al. 2012) and events (Guazzi, Reina et al. 2009). The chapters 3, 5, 6 and 7 utilized non-invasive flow mediated dilatation on the brachial artery for predicting risk and measuring function.

#### 1.4.10 Measurement of Endothelial Dysfunction

There are different techniques to evaluate the endothelium functional capacity, that depend on the amount of nitric oxide NO produced and the vasodilatation effect. Most of the functional methods for *in vivo* endothelial testing examine the ability of the endothelium to cause vasodilation in response to pharmacological and physiological stimuli that increase the endothelial release of NO. The percentage of vasodilatation with respect to the basal value represents the endothelial functional capacity. Taking into account that shear stress is one of the most important stimulants for the synthesis and release of NO, the non-invasive technique most often used is the transient flow-modulated "endothelium-dependent" post-ischaemic vasodilatation (FMD), performed on conductance arteries such as the brachial, radial or femoral arteries. This vasodilatation is compared with the vasodilatation produced by drugs that are NO donors, such as nitroglycerine, which is called "endothelium independent". The vasodilatation is then quantified by measuring the arterial diameter with high resolution ultrasonography (Celermajer, Sorensen et al. 1992). The technique most commonly used, at present, involves creating shear stress in the forearm vessel (the brachial artery) by applying a transient compression in the forearm, up to 200 mmHg, using a pressure cuff. This creates an artificial transient ischaemia in the down-stream brachial artery. Once the pressure from the cuff is released, typically after 4 to 5 minutes, the blood flow in that section of the artery rapidly increases, likely as a response to the post-ischaemic environment. This increased flow creates sheer stress and, in turn, stimulates NO release that causes vaso-dilatation. This is the underlying mechanism of non-invasive flow mediated endothelial dependant (FMD) vasodilatation of the brachial artery (Celermajer, Sorensen et al. 1992; Corretti, Anderson et al. 2002; Esper, Nordaby et al. 2006).



## **1.5 Microvascular Function**

### **1.5.1 Definition**

Micro vascular function is the orderly functioning of smaller conductance and resistance vessels, of the size 100 to 200 microns, which play a role in the regulation of blood flow and micro-circulation.

### **1.5.2 Anatomy and Physiology of Retinal Vessels**

The micro-circulation of the retina is composed of arterioles, capillaries and venules. Arterioles are small resistance vessels (20-150  $\mu\text{m}$ ) composed of an endothelium surrounded by one or more layers of smooth muscle cells. The function of the arterioles is to regulate how much blood is delivered to the downstream capillary bed. The diameter of the arterioles depends on the contractile state, primarily of the smooth muscle, which is regulated by multiple factors, such as endothelium derived constricting factors, endothelium derived relaxing factors (EDRF) and circulating vasoactive hormones. Capillaries (6-10  $\mu\text{m}$  in diameter) are single celled endothelial cells surrounded by a basement membrane, presenting without a smooth muscle layer. Continuous capillaries are present in the retina and are the most impermeable type, resulting in the formation of the blood retinal barrier, similar to that of the brain-blood barrier. Physically, the first barrier between blood and tissue in the retinal circulation are endothelial cells, which prevent plasma from leaking out of the vessel and are important in the autoregulation of the blood flow. The endothelium consists of a monolayer of cells covering the inner wall of the vasculature and is strategically located between the circulating blood and the vascular smooth muscle cells (Haefliger, Flammer et al. 2001). The second vascular layer is the smooth muscle which can constrict and dilate; these cells are

enclosed by an internal and an external elastic lamina. The internal elastic lamina is located between the smooth muscle cells and the endothelium and transmits signals between the two layers. The outer layer protects the vessel and attaches the vessel loosely to the surrounding tissues (Levick 2000). In summary, retinal arterioles are composed of endothelial cells and smooth muscle whereas retinal capillaries are made up of tight, non-fenestrated endothelial cells surrounded by a basement membrane containing pericytes and mural cells. The endothelial cells are joined together by zonula occludens, thereby preventing leakage from the arterioles, capillaries and venules and preserving optimal retinal function (Remington 1998 ).

### 1.5.3 The Vascular System of the Retina

The eye obtains its blood mainly from the ophthalmic artery, which is the first branch of the internal carotid artery and the only branch of the internal carotid outside the cranium (Harris 2nd Edition, Elsevier, 2010. ). The adult neural retina is supported by two distinct vascular systems, the inner retinal vessels and the choroidal vessels. The two beds vary in both their embryonic differentiation pattern and functionality in the adult organism. The retinal vasculature has barrier properties similar to those observed in the brain, whereas the choroidal vessels demonstrate a greatly fenestrated phenotype (Saint-Geniez and D'Amore 2004).

#### 1.5.3.1 The Retinal Blood Vessels

The retinal blood vessels nourish the inner layers of the retina. The inner retina maintains its blood via the aortic artery, common carotid arteries, internal artery, ophthalmic artery and, finally, the central retinal artery (CRA). The CRA enters the optic nerve 10-15 mm behind the globe and runs forward in the central section of the nerve along the central retinal vein. CRA

supplies the inner two third of the retina, the most anterior portion of the superficial nerve fibre layer of the optic nerve head and, to some extent, the retro-laminar optic nerve.

#### 1.5.3.2 The Choroidal Vessels

The outer retinal layers, including the photoreceptors, are nourished by the choroid. The uveal system, specifically the chorio-capillaries, supplies the deeper layers, including photoreceptors and bipolar cells. The short posterior ciliary arteries directly supply the choroid and the long posterior ciliary arteries travel in the supra-choroidal space anteriorly to supply the choroid anteriorly via recurrent branches.

In summary, retinal tissue is nourished by two vascular systems of blood vessels that differ anatomically and physiologically. The inner retina is nourished by the distribution of the central retinal artery. The outer retina is nourished by the underlying chorio-capillaries via the short and long posterior ciliary arteries. Blood is drained by the retinal venules into the central retinal vein and finally the ophthalmic vein.

The retina is able to regulate blood flow in response to different metabolic demands. There are several animal and human studies demonstrating a change in retinal vessel diameters (Polak, Schmetterer et al. 2002; Dorner, Garhöfer et al. 2003; Dorner, Garhofer et al. 2003) as well as in retinal (Michelson, Patzelt et al. 2002) and optic nerve head blood flow (Buerk, Riva et al. 1996; Riva, Falsini et al. 2001; Falsini, Riva et al. 2002) as a result of provocation with diffuse luminance flicker, O<sub>2</sub>, CO<sub>2</sub>, etc. Like in the brain, blood flow in retinal vessels is strongly coupled to neural activity, which has been recently established using laser Doppler flow metry (Buerk, Riva et al. 1996) and retinal vessel diameter measurements (Formaz, Riva et al. 1997; Dorner, Garhöfer et al. 2003; Dorner, Garhofer et al. 2003). Hemodynamics

is the study of the properties and mechanisms that control the flow of blood. Blood flow within an organ or vascular network is driven by the difference between the arterial and venous pressures across the organ which is termed perfusion pressure (i.e. pressure gradient). The vascular smooth muscle cells have an intrinsic ability to either constrict or dilate in response to increase or decrease of the perfusion pressure, respectively. The smooth muscles respond by contracting when the lumen of a blood vessel is suddenly expanded, as occurs when intravascular pressure is suddenly increased. Conversely, a reduction in intravascular pressure results in vasodilation. Therefore the arterioles respond to intravascular pressure as a stimulus (Johnson 1986).

#### 1.5.4 Diabetic retinopathy

Diabetic retinopathy is a highly specific micro-vascular complication of diabetes and the leading cause of blindness in people under the age of 60 in industrialised countries (Sibal and Home 2009). It is also a major cause of blindness in older people. At 20 years from the initial onset of diabetes, over 90% of people with Type 1 diabetes and more than 60% of people with Type 2 diabetes will have diabetic retinopathy (Sibal and Home 2009). Due to the sight threatening potential of this disease, and the availability of methods to slow down the rate of progression of the disease pathogenesis, the UK National Screening Committee has recommended screening for diabetic retinopathy as a national priority (Sibal and Home 2009) which is the current practice now. Whilst the mechanisms underlying diabetic retinopathy are unclear, the progression of the disease is well known and may take several forms.

The initial stage is background retinopathy which is characterised by capillary micro-aneurysm formation, dot and blot intra-retinal haemorrhages and lipid exudates. This stage of the disease does not usually impair any visual function. The next stage of the disease process,

labelled, pre-proliferative retinopathy, is characterised by capillary and arteriolar closure. The late stage in this process leads into proliferative retinopathy which is characterised by the growth of new blood vessels (Figure 1.5.4). This stage commonly leads to progressive visual impairment caused by scarring and bleeding (Acharya, Chua et al. 2008).

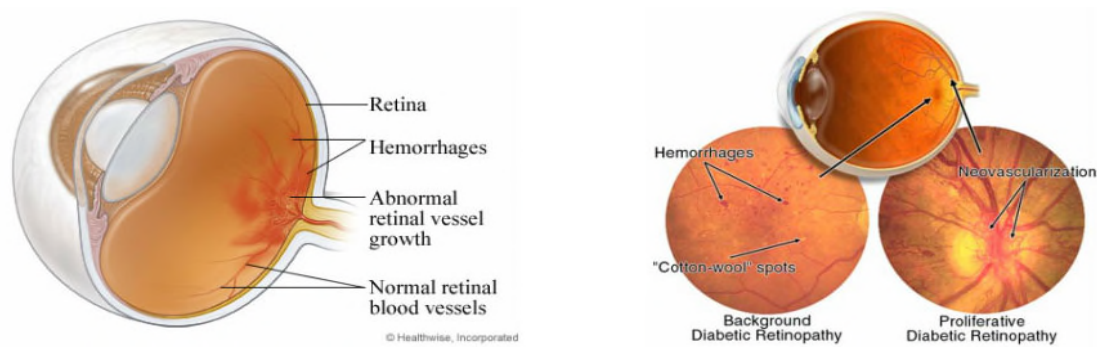


Figure 1.5.4 Different stages of Diabetic Retinopathy.

Pooled data from many of the available screening studies up to 1996, in their modelling project on the effectiveness of diabetic retinopathy screening programmes, have determined that retinal photography was the screening test of choice (Sibal and Home 2009). Background retinopathy in women predicted CVD and CHD death. These associations have been shown to be independent of current smoking, hypertension, total cholesterol, glycaemic control of diabetes, duration of diabetes, and proteinuria (Juutilainen, Lehto et al. 2007). A prospective study involving Type 1 DM patients with retinopathy clearly revealed an increased risk for all cause mortality and incident CVD (van Hecke, Dekker et al. 2005). A more recent study in 2015 investigating the association of diabetic retinopathy with the risk of cardiovascular disease in type 2 diabetic patients revealed a clear association (Shoeibi and Bonakdaran 2015). One-hundred and eighty patients with type 2 diabetes without CVD at baseline were enrolled in that study. Patients were classified into three categories: no diabetic retinopathy (NDR), nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic

retinopathy (PDR) based on fundoscopic findings. Ten-year CVD risk was estimated using the United Kingdom Prospective Diabetes Study risk engine. Patients were classified as high risk ( $>20\%$ ), moderate risk ( $10\text{--}20\%$ ) and low risk ( $<10\%$ ). The total prevalence of DR was  $30.5\%$ . After adjustment for traditional risk factors for CVD, the risk of CVD remained markedly increased in the presence of diabetic retinopathy (Shoeibi and Bonakdaran 2015).

#### 1.5.5 Retinal vessel Analysis on Retinal Vessel Analyser (RVA)

The RVA comprises a optic fundus camera (model FF 450; Zeiss, Jena, Germany), a video camera, a real-time monitor, and a computer with image-analysis software for the accurate determination of retinal arterial and venous diameter. Retinal vessel diameter was analyzed in real time with a maximum frequency of 40 Hz. Retinal vessel reactivity (RVR) was measured using the retinal vessel analyser (RVA, Imedos, GmbH). The RVA system was most suited to undertake quantitative investigation of retinal vasosclerosis, especially in association with arteriosclerotic disease. Using RVA system, diameter changes in retinal arterioles was first reported in a clinical methodological study by Nagel (Nagel and Vilser 2004). The RVA measured the diameter of the column of the red blood cells. After the fundus camera was adjusted to the dilated pupil, a vessel section of the eye, measuring approximately 1.5 mm in length, was evaluated. Retinal vessel diameter was analysed in real time with a maximum frequency of 40 Hz, so that every second a maximum of 25 readings of vessel diameter could be obtained. For this purpose the fundus was imaged onto the charge-coupled device (CCD) (camera with an apparatus which was designed to convert optical brightness into electrical amplitude signals using a plurality of CCDs. It then reproduced the image using the electric signals without time restriction) chip of the video camera (Appendix I, Figure 1 h ). The consecutive fundus images were digitized with a frame grabber. The vessel diameters were measured with the software, Vesselmap 2 (IMEDOS) (Mandecka, Dawczynski et al. 2007;

Nagel, Vilser et al. 2007). Evaluation of the retinal venous and arterial diameter was performed using above software in a “Live” format ( real time) instantly. These pictures could also be reviewed and re- analyzed retrospectively, from the recorded videotapes (Seifertl and Vilser 2002). The position of the vessel edges was determined and the angle and diameter of the vessel was calculated automatically, online. After baseline recording of 50 seconds, three flicker periods of 20 seconds each was applied, interrupted with 80 seconds of still illumination. The point of maximum dilation was determined for each period of flicker stimulation as the highest 1-second mean vessel diameter during the period of 50 seconds. A graph was generated which described the capability of the vessel to dilate. This function is highly standardized and thus applicable in clinical routine (Nagel and Vilser 2004).

#### 1.5.6 Functional Measurement of Micro-circulation and Utility

The RVA system has the ability to reliably measure the vascular reactivity of the micro-circulation, the retinal vessels. Dynamic Vessel Analysis (DVA) is the key technology for the early detection of functional vessel changes. Dynamic microvascular changes in response to stress can be reliably and consistently compared between groups. Therefore this method of RVA analysis might be sensitive enough and may prove to be a valuable research tool and source of data analysis for assessing the impact of the post-prandial state on the risk of CVD and retinopathy in T2DM (Nagel and Vilser 2004; Gugleta, Zawinka et al. 2006).

### 1.6 Comparison with Macro vascular Function

Microvascular disease, by definition, is disease of the small and finer blood vessels in the body, including the capillaries. By contrast, macrovascular disease describes disease of the

large blood vessels including the aorta, as well as the coronary arteries and major arteries of the limbs and the brain. Until recently, the effects of hyperglycaemia, physiological stress and its effects on macrovascular function, resulting in cardiovascular risk, have been clearly understood and published. However, similar physiological explanations, with regard to stressors and cardiovascular risk, have not been proven in microvascular endothelium. Our previous publication (Chittari, McTernan et al. 2011) defines the functioning of endothelium at both macrovascular and microvascular levels, simultaneously. For the first time, we can reveal that physiological alterations inside the lumen of blood vessels could be detected immediately at both vascular beds. This detection leads to a simultaneous but a varied and divergent response from both macro- and microvascular endothelium.

#### 1.6.1 Brachial Artery Flow Mediated Dilatation (FMD)

Endothelial dysfunction can be detected in the forearm as an impairment of endothelium dependent flow mediated vasodilation, even before the development of clinical atherosclerosis. Atherosclerosis is common in the human brachial artery and is significantly correlated with both coronary and carotid disease. These results suggest that the brachial circulation may serve as a reasonable "surrogate" for studying atherosclerosis, particularly in younger adults (Sorensen, Kristensen et al. 1997).

#### 1.6.2 Vascular function predicting Cardiovascular disease

Endothelial dysfunction, by these methods, correlates with cardiovascular risk factors (Chan, Colhoun et al. 2001), and may be predictive of incident cardiovascular events (Perticone, Ceravolo et al. 2001; Lerman and Zeiher 2005; Yeboah, Crouse et al. 2007). Brachial artery



median FMD independently predicts long-term adverse cardiovascular events in healthy subjects in addition to traditional risk factor assessment (Shechter, Issachar et al. 2009). The endothelial function of the brachial artery measured by this FMD method has shown a good correlation with the coronary artery response implicating the brachial artery as a "surrogate" for the coronary artery (Anderson, Uehata et al. 1995; Sorensen, Kristensen et al. 1997). An excellent correlation has been noted with the severity of the vascular lesions, even when they are asymptomatic and undetectable, by the usual clinical methods. It has also been reported that patients without CVD but with risk factors, had a diminished brachial artery vasodilatation response when compared with a normal population. Furthermore, if they had peripheral artery disease, the response was even more diminished compared with the positive risk factor population. This leads us to the assumption that, as the severity of the lesions increases, so does the endothelial dysfunction (Yataco, Corretti et al. 1999; Esper, Nordaby et al. 2006). Therefore attenuated brachial artery FMD correlates with the presence of coronary endothelial dysfunction and atherosclerosis, as assessed by angiography, highlighting the systemic nature of vascular disease (Anderson, Uehata et al. 1995) and demonstrating that the technique is both reproducible and reliable (Sorensen, Celermajer et al. 1995).

The FMD technique in the brachial artery could also be used to monitor treatment benefit (Ceriello, Taboga et al. 2002), in addition to its value in predicting future CVD. FMD as a tool, also appeared to have been utilised to independently predict postoperative cardiac events in patients undergoing vascular surgery (Gokce, Keaney et al. 2002). A study from Japan published in 2006 showed that cardiac patients who had low values for the FMD brachial artery had twice the number of cardiac events on 5 year follow up compared with higher FMD values (Yoshida, Kawano et al. 2006).

## 1.7 Aims of thesis

Whilst it is clear that endothelial dysfunction in obesity, IGT and T2DM is an important primary event leading to increased risk of developing CVD, the non-invasive vascular assessments in different peripheral vessels and vascular beds creating varied risk are not fully established or understood. With increasing prevalence of these risk groups across the world, it is becoming very clear that primary prevention in the way of screening and identifying the risk groups within this population should pay dividends in the form of reduced incidence of CVD.

Therefore, understanding the molecular (biomarkers) and physiological mechanisms of endothelial function at different vascular beds will help in identifying the early features of the disease process and, in turn, provide an opportunity to initiate strategies for its reversal. Finally, effect of Metformin therapy in diabetic patients will be assessed on its effect on biomarkers and in turn cardiovascular risk.

This thesis will, therefore, aim to:

1. Study the impact of diet, specifically acute hyperglycemia and acute hyperlipidemia on serum biomarkers of inflammation and endothelial dysfunction including TNF- $\alpha$ , Leptin and Endotoxin. The acute effects of hyperglycemic and hyperlipidemic will also be studied on the functioning of the endothelium at the macrovascular level in healthy subjects and compare and contrast it with CVD risk groups (T2DM and Obesity including obesity in children).
2. To study prospectively the microvascular function as measured by Microvascular reactivity at the level of retina in healthy subjects and above high risk groups using physiologic stress as detailed above.

3. For the first time, compare and contrast both modalities (microvascular and macrovascular function) in healthy subjects and high risk groups in their ability to measure endothelial dysfunction.
4. Finally, investigate the protective effects of Metformin therapy on lipoprotein damage in T2DM subjects compared with healthy subjects.

## **Chapter 2**

### **General materials and methods**

## **2.1 General materials and methods**

This section covers in detail all the methods that are used in this thesis. More detailed information regarding any specific methodology used in a particular chapter will be covered in more detail in that relevant chapter. Further details on specific detailed methodology will be covered in appendices at the end of the thesis.

### **2.1.1 Patient selection and recruitment**

The recruitment of subjects for the study was conducted both in primary care centres/general practices as well as University Hospital Coventry and Warwickshire (UHCW) NHS Hospital, in Coventry. The general practice surgeries which were participating in our study identified the patients who would qualify for the study, (depending on detailed inclusion and exclusion criteria) from their databases. Once they were identified, all qualifying patients were invited to attend the research clinic. They were contacted by telephone to confirm their willingness to partake in a research project, which could potentially be relevant to them. After they had given consent to participate, patient information sheets (Appendix I, 1a) were posted to their home address. The subjects were subsequently contacted and were invited to the research clinic following a strict 8-12 hour overnight fasting, which all subjects re-confirmed on the day.

### **2.1.2 Research Clinic**

The research clinic was conducted at a General practice surgery which was participating in our study. This surgery was central to the population in the study. The clinic was run,

initially, once a week and subsequently twice a week until the full number of subjects was recruited. The clinic was staffed by the research doctor and two female research support nurses who assisted in patient contact, logistics of posting letters and smooth running of clinics, in addition to assisting with blood pressure recordings and electrocardiographic recordings. They were also used as chaperones to ensure patient confidence and assistance.

### 2.1.3 Selection criteria

Patients were identified on the database after matching for strict criteria, as detailed below. Age criteria were used in this study in view of mounting evidence of advancing age influencing and, in most circumstances, depressing the endothelial function (SIEKER and HICKAM 1953; Celermajer, Sorensen et al. 1994; Gerhard, Roddy et al. 1996).

#### 2.1.3.1 Inclusion criteria

Type 2 diabetes mellitus (T2DM) less than 65 years old.

Non diabetic subjects (ND) less than 65 years old.

#### 2.1.3.2 Exclusion criteria

Acute illness

Pregnancy

Uncontrolled hypertension

Any history of vascular disease [including Cerebrovascular accidents (CVA), Transient ischaemic attacks (TIA), Myocardial infarction (MI), Ischaemic heart disease (IHD), Peripheral vascular disease (PVD), Intermittent claudication (IC) etc]

Abnormal resting Electrocardiogram (ECG)

Smoking

Subjects taking aspirin, statins or supplemental vitamins

Body mass index (BMI) > 50

## **2.2 Clinic Visit**

The initial clinic visit was undertaken in the mornings to facilitate taking fasting blood tests. Some clinics were also conducted in the afternoon for subsequent visits. Once at the clinic, the study was explained to the subjects' in the appropriate language, with the use of interpreters when it was deemed necessary. After confirming that the study was fully understood, participating subjects had an opportunity to ask further questions; following this they signed an informed consent form (Appendix I, 1 b). After enrolling into the study, fasting blood and urine samples were obtained. The blood tests conducted included renal function tests, blood glucose, glycated haemoglobin (HbA1C), full cholesterol profile and C-reactive protein (CRP). These tests were analyzed at the UHCW biochemistry laboratory within the hospital. Baseline demographic profile was obtained including relevant history using a standardized proforma and checklist (Appendix I, 1 c). All subjects had their height, weight, abdominal circumference and body mass index (BMI) measurements taken using the same equipment to standardise measurements. Blood samples were taken, either from the

right or left ante-cubital vein, in a sitting position. Blood pressure was checked with a Blood pressure (BP) monitor (Omron Intellisense M7 BPM Blood Pressure Monitor with Multi Cuff) on the left arm and re-checked after 2 min. Finally a 12 Lead ECG was performed using the same machine (Seca CT3000i) for the duration of the study. Female chaperones were present during the clinics for female subjects.

The control group of healthy ND subjects was identified in the same manner as subjects with T2DM, by the same practice managers of the General practice. The criteria used to identify these subjects were identical. Subjects in this group were matched for age, gender, ethnicity and BMI of the already enrolled diabetic population. Again, only non-smoking subjects with a normal resting ECG, normal BP and no history of vascular disease were selected. Subjects on aspirin, lipid-lowering agents, or supplemental vitamins were excluded as they can influence endothelial function. All subjects, both T2DM and ND, were recommended to consume their habitual diet during the entire period of the study. On the first day of physiological testing, a validated dietary questionnaire was used to obtain dietary habits of all participants. (Appendix I,1d). This copy righted product from Tinuviel software was purchased with Research funds.

At the end of the visit to the clinic, patients were offered a drink prior to leaving. A telephone appointment was booked a few weeks before the start of the physiological tests. They were asked to attend the UHCW first thing in the morning on the day of their appointment, preceded by an 8 hr overnight fast. As detailed in the patient's information sheet (Appendix I, 1 a) and study protocol, (Appendix I, 1d), the subjects attended the UHCW on 3 different days, each day following an overnight fast of a 8 hr minimum. The tests were finished by lunchtime, as detailed in Appendix 1I, figure 2 a.



### 2.2.1 Patient Recruitment

In order to recruit naive T2DM subjects, efforts were made to screen and identify at-risk people, not only at participating surgeries but also at non-traditional places like health awareness camps and at cultural fairs. At-risk groups were defined as people with a family history of ischaemic heart disease and T2DM, obese people and people of South Asian origin. After informed consent and the same standardized procedure, this healthy group was similarly recruited into the study.

### 2.2.2 Serum and plasma isolation

The following procedure to isolate serum and plasma was immediately performed at the clinic visit. Once blood was drawn into separate tubes, the tube collecting serum was spun immediately in a centrifuge (Capricorn Laboratory Equipment, model CEP2000) at 3000 revolutions per minute (RPM) ( $1500\times g$ ) for 15 min. Then the tube was placed upright in ice until it reached the research laboratory. The tube collecting plasma was left to clot for 20 min and subsequently spun in a centrifuge at  $1500\times g$  for 15 min and then this tube was also placed on ice until it reached the research laboratory. Similarly, the two tubes containing whole blood and urine, respectively, were appropriately stored in a cold container until it reached the storage freezers in the laboratory based at Clinical Services Research Institute Laboratories (CSRI) at University Hospital Coventry. Once at the laboratory, all samples were appropriately coded, fully anonymised, labelled and stored at  $-80^{\circ}\text{C}$ .

## **2.3 Physiological Tests**

### **2.3.1 Study Outline**

Physiological tests were performed at UHCW over a period of 3 days. Patients were required to attend all 3 days, preferably, consecutively, where they were subjected to a glucose challenge as part of an oral glucose tolerance test (OGTT) on day 1, fat stress on day 2 and lastly combined stress involving both oral glucose and fat stress on day 3 (Ceriello, Taboga et al. 2002; Ceriello, Quagliaro et al. 2004). All subjects were required to complete the 3 days of the studies within 4 weeks of the first day of the study. On the day of the test, the subjects attended the UHCW centre early in the morning, after an 8 hr overnight fast. T2DM subjects omitted their morning insulin or metformin medication, as they were fasting. Patients on anti hypertensives omitted their previous night's dose and all subjects were advised to bring their medications with them on the morning of their tests. Once at the study centre (Appendix I, Figure 1 f), patients' details, date of birth, fasting status, drugs history, consent and signature were confirmed. Following this, the patients were given a further detailed briefing about the study protocol and procedures (Appendix II, Figure 2 a) to ensure they were comfortable to proceed with the study.

### **2.3.2 Study protocol**

Blood samples were drawn at 0 (fasting state), 1 (1 hr post-stress), 2 (2 hr-post stress), 3, and 4 hr-post stress. Glucose, full lipid profile, insulin, inflammatory and endothelial markers were analyzed for each time period. Along with the blood tests, endothelial function, at the level of the arm and retina, was measured. Ultrasound technique to measure flow-mediated vasodilatation (FMD) of the brachial artery and retinal vessel analyser (RVA), to measure

retinal vessel reactivity (RVR), was undertaken at same the time points, along with the blood tests. On the first day, standard OGTT with 75g glucose was consumed as physiological stress for measuring endothelial function. The next day high fat meal consisting of 158ml of double whipping cream (ASDA supermarket) standardized to contain 75g of fat was consumed before physiological testing. On the final day both glucose and fat, at the same doses as previously described, were given as a meal (Appendix I, study protocol 1 e). The nutritional value of the double cream is shown below.

Typical values	Per 100ml
Energy	1831kJ
	445kcal
Protein	1.7g
Carbohydrate	2.6g
of which sugars	2.6g
Fat	47.5g
of which saturates	29.7g
Fibre	Nil
Sodium	Trace
equivalent as salt	0.1g

Table 2.3.2 ASDA fresh double cream: Nutritional content.

### 2.3.3 Endothelial Function at Macrovascular level

Endothelial function was evaluated by measuring flow-mediated vasodilatation (FMD) of the brachial artery. It was a non-invasive method of assessing the endothelial function at the macrovascular level. Endothelial dependant vaso-reactivity has been a routinely used clinical endpoint for assessment of endothelial function (Deanfield, Halcox et al. 2007). Endothelial dysfunction reflective of cardiometabolic changes appears to be an early risk marker for catastrophic CV events (Gupta, Ravussin et al. 2012). FMD brachial artery scan measurements were considered as surrogate for atherosclerosis and therefore the foundations

of cardiovascular risk (Anderson, Uehata et al. 1995; Sorensen, Kristensen et al. 1997). The examination was carried out in a temperature and light controlled room on subjects who were lying comfortably flat on a couch (Appendix I, Figure 1 f). The subject's left arm was resting by their side whilst the right arm was placed comfortably on a height adjustable special table (Appendix I, Figure 1 f). In this study, the temperature in the study room was constantly maintained at 21°C using an efficient central air conditioner. The room temperature was monitored daily using a room thermometer, apart from occasional cross checking using an efficient sensor and storage device (this continuously tracks the temperature by the minute for a period of time which then gets downloaded to a computer to analyze accurate findings). The conditions in the room were kept as quiet as possible with only researchers and subjects present. Usually, there were two subjects, 2 operators and another female research assistant as a chaperone. A BP cuff was placed on the left arm which was connected to an automated quality checked and a National Health Service (NHS) approved BP machine (Model DASH 3000- GE Medical systems) which was used to check systolic and diastolic BPs at various times during the study protocol. The same machine was used from the beginning to the end of the study, to reduce any possible variations between machines.

ECG tracing was obtained by 3 leads connected to the VIVID 7 ECHO machine (GE Vingmed System V) (Appendix I, 1 g). Similar tracing on the studied subjects was used to monitor the heart rate continuously throughout the study. A 12 MHz flat linear array probe was connected to the Ultrasound machine which was used to scan a longitudinal section of the brachial artery. The probe was held in the same position during the scan, using a specially designed stereo-tactic clamp. The clamp was used to position the relaxed, rested, right arm in place from the beginning to the end of the study. An electronic dual tourniquet microprocessor device was used to instantly inflate an appropriate cuff placed on the distal arm below the transducer position. The study was designed to measure endothelial function

proximal to the pressure cuff. This was in contrast to other methods, such as the upper arm pressure cuff and distal scanning. The forearm compression was the favoured approach not only in guidelines (Corretti, Anderson et al. 2002), but also because it held statistical advantages over upper arm compression (Peretz, Leotta et al. 2007). Forearm compression was also more likely to represent NO mediated endothelial response than upper arm proximal compression (Tschakovsky and Pyke 2005). This tourniquet device inflates the cuff to supra-systolic pressures, typically 200mmHg to produce ischaemia in the forearm. The cuff was then deflated after 5 min thus causing a reactive hyperaemia, which, in turn, produced a shear stress stimulus that induces the endothelium to release NO, a vasodilator. FMD, which reflects endothelium dependent vasodilatation, was calculated as the percentage increase in diameter from baseline to the value obtained at 50 sec after the cuff deflation.

#### 2.3.4 Flow mediated dilatation (FMD) Measurements

FMD is an established non-invasive technique for assessing endothelium function using high resolution ultrasonography. Endothelial function in this study was evaluated by measuring FMD of the brachial artery. The traditional method of measuring FMD broadly follows the technique as described below, by Celermajer in 1992 (Celermajer, Sorensen et al. 1992). A more recent systematic review in 2012 appears to indicate that brachial artery FMD predicts cardiovascular risk (Garcia, Lima et al. 2012). Vasodilatation responses of the brachial arteries are usually measured using ultrasound technique by a qualified examiner. The diameter of the brachial artery was measured from B-mode ultrasound images with a 7-12 MHz linear array transducer probe. Flow velocity in the brachial artery was measured with a pulsed Doppler signal at a 70° angle to the vessel. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Depth and gain settings were optimized at the

beginning of the study and were kept constant throughout the recording period. When a satisfactory transducer position was noted, the surface of the skin was marked, and the arm remained in the same position throughout the study. Each subject lay quietly for 10 min before the first scan. At the end of each test, the subjects lay quietly for 15 min. Finally, sublingual nitroglycerin (GTN) was administered and 3 min later, the last measurements were performed. Response to GTN was used as a measure of endothelium-independent vasodilatation. All studies were performed in a quiet and temperature controlled room (22°C to 23°C).

After baseline measurements of the diameter and flow velocity including pulse wave Doppler in the brachial artery were obtained, a sphygmomanometer cuff placed around the forearm was inflated to a pressure up to 200mm Hg and released after 5 minutes. The arterial diameter was continuously monitored and recorded during the whole scanning time. The ultrasound images were recorded on a VHS video cassette recorder and the arterial diameter was measured with ultrasonic calipers, by two independent qualified observers (M. C. Corretti 1995). Diameter measurements for the reactive hyperemia were taken 45 to 90 sec after cuff deflation to measure peak diameter. Responses of the vessel diameters to the reactive hyperaemia and GTN were expressed as the percent increase above the baseline value of the diameter. Blood flow was calculated by multiplying the velocity-time integral of the doppler flow signal by heart rate and the vessel cross-sectional area. The increase in the blood flow was calculated by dividing the maximum flow within the first 15 sec after the cuff deflation by the flow at baseline (M. C. Corretti 1995).

As this technique had a potential to involve significant observer variability, guidelines were created in 2002 to standardise the technique (Corretti, Anderson et al. 2002). Even though this improved the issue of variability, the procedure still continued to have significant drawbacks

which limited the confidence of values and results obtained from using the above described technique. Retrospective analysis, in short, gave rise to several problems (Newey and Nassiri 2002) which are discussed in more detail in later chapters.

Many of these potential problems were resolved when the data was analyzed online (Newey and Nassiri 2002). The system used for this study allowed live on-line analysis with a single operator. The tracking and optimisation for most part was done automatically by the system software. In view of live analysis, high image acquisition rate was possible and results were obtained instantly. This obviated the need for retrospective analysis. Finally, this system did not require the need for continuous ECG monitoring for cardiac cycle changes, as it could analyse changes of cardiac cycle from monitoring the arterial wall motion. Artery diameter was calculated by detecting the vessel walls within a user-defined image region, using an automated computer algorithm. These automated systems were developed to overcome the specific problems associated with previous retrospective data analysis and to improve the evaluation of FMD from ultrasound data (Newey and Nassiri 2002).

This study utilised all the accepted criteria for best acquisition of FMD images. The usage of these criteria resulted in an effective and reliable NO mediated response (Pyke and Tschakovsky 2005). The criteria included distal placement of the occlusion cuff to the site of FMD measurement, 5 min or less occlusion duration and obviating the need for ischaemic hand grip exercise for measuring FMD of brachial arteries (Pyke and Tschakovsky 2005). This software was loaded onto a personal computer and connected to a standard ultrasound machine using a frame grabber. The network design and technology involved in designing such an on-line system was clearly described by Newey in 2002 (Newey and Nassiri 2002). This system involved using specially designed software on a personal computer that automatically located the correct vessel walls within a user-defined region-of-interest (ROI)



and measured vessel diameter online throughout an FMD study. The system was designed to minimise operator workload. The only user input normally required was to drag an orthogonal ROI over the required vessel section and to press a start button (Newey and Nassiri 2002).

Another important dimension to the newer system was the wall tracking ability. The tracking system was designed to identify the vessel walls in the first image by searching the entire ROI pixel-by-pixel with each network in turn, with the input line section centered on the current search location. This search generated matrices containing the probability of each ROI point being a vessel wall. The matrices were examined to select the most likely vessel wall starting points, using a range of criteria. These included the wall probability level, wall order (*i.e.* anterior before posterior), wall separation distance and the mean intensity levels above and below the starting points. Walls are then propagated laterally from the starting points to create a vessel wall pair. Lateral propagation consisted of a small sideways step followed by a vertical search with the appropriate network to find the point at the new step with the highest probability of being a wall. This process was repeated from the highest probability point at each step until the edges of the ROI were reached. The detected walls were then overlain on the image and then vessel diameter was calculated relative to each point along the vessel axis. The highest probability point on each wall was then used as the focus for wall propagation in the following image (Newey and Nassiri 2002). The tracking process was entirely automatic, except for selection of the ROI. Controls were provided for minor ROI adjustments, and wall tracking could be manually overridden on-line by pointing and clicking.

In FMD studies, it was necessary to measure artery dilatation changes at diastole. This was undertaken to avoid inaccurate and inconsistent measurements when the cardiac cycle was not taken into account. This required synchronisation of the scanner image to one point in the

ECG. The scanner image was, therefore, only updated once in every heartbeat so that information that was potentially useful (*e.g.* in vessel elasticity studies) was not lost. In the study, by continuously updating the scanner image and measuring the vessel diameter at 25 images every second, it was possible to recover vessel diameter changes over the cardiac cycle as the vessel expanded and contracted with BP changes. Cardiac events were detected using a cross-correlation search technique that located the most highly correlated events. The detected events were averaged after normalisation to heart rate, and the search was directed to maximise the systolic-to-diastolic difference by fine-tuning the correlation window width and position. The diameter changes over the cardiac cycle could be recovered reliably by this method (Sidhu, Newey et al. 2002).

The above described method of brachial artery imaging could represent this technique as a gold standard for clinical research on conduit artery endothelial biology like the brachial artery (Deanfield, Halcox et al. 2007). It is highly likely future studies would be based on similar instant semi-automated analysis rather than operator dependant off line retrospective video editing methods. In this study, brachial arteries were imaged with a standard ultrasound system (VIVID 7 ECHO machine (GE Vingmed System V) connected with a 12 MHz linear transducer probe. The ultrasound system was connected to a personal computer equipped with a frame grabber and artificial neural network wall detection software (vessel image analysis) (VIA) (Sidhu, Newey et al. 2002), as described previously. This system was developed by Val Newey's group at St. George's Hospital, London. The VIA software automatically detects and tracks the anterior and posterior walls within a user defined region of interest (Figure 2.3.4.1 below) (Sidhu, Newey et al. 2002).

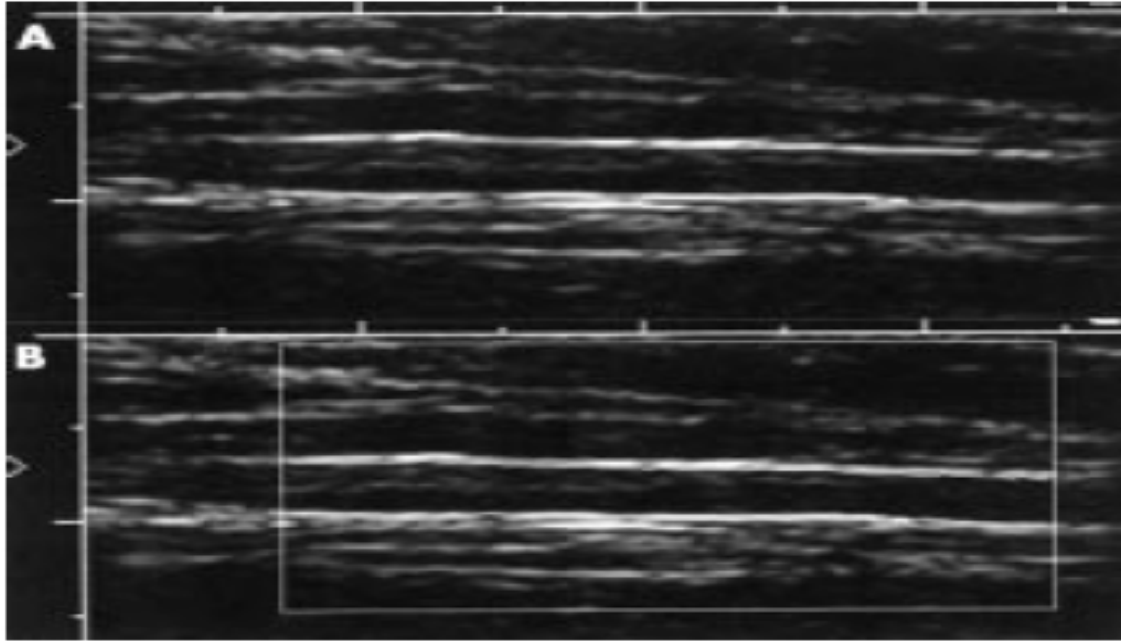


Figure 2.3.4.1 VIA software detecting anterior and posterior walls in the ROI (Sidhu, Newey et al. 2002)

The white box in the bottom half of the picture was the ROI. The brachial artery walls were clearly visible within the ROI as two parallel white lines across the picture, which corresponds to anterior and posterior walls.

The software could also accommodate angulations of the artery (up to  $20^\circ$ ) relative to the perpendicular. The B mode images were processed at 25 frames every sec and the vessel diameter, including diameter changes over the cardiac cycle, was displayed in real time (Figure 2.3.4.2) (Sidhu, Newey et al. 2002).

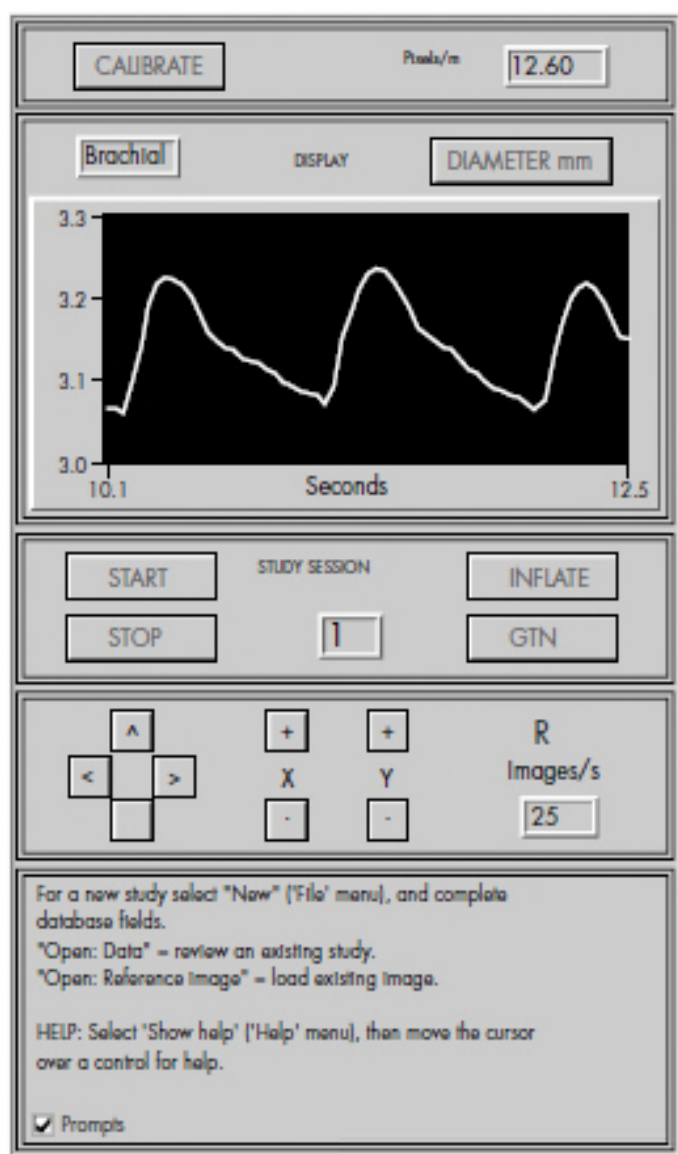


Figure 2.3.4.2 VIA software displaying real time diameter changes over cardiac cycle (Sidhu, Newey et al. 2002).

The image reveals the acquisition rate of pictures from the ultrasound machine to the computer with VIA software for this analysis. This is depicted as pixels per min. The display on the figure shows cardiac cycle changes as a monitored graph.

This method of instant and accurate monitoring of the endothelial boundaries allowed ultrasound imaging parameters to be optimized at the start of the scan and the transducer position to be adjusted immediately for optimum tracking performance during the entire test.

The brachial artery was scanned longitudinally 2–10 cm above the elbow, until the clearest possible image of the anterior and posterior wall media was obtained. Depth and gain settings were kept constant during the rest of the study. A custom made stereotactic clamp was used to hold the transducer in a fixed place and a screw gauge was used to make small compensatory movements of the transducer to accommodate subject movement during the test (Appendix I, 1 f). The distance of the probe from the antecubital fossa was noted and a two dimensional image of the brachial artery was saved on the computer's hard drive for reference in repeat studies. Brachial artery FMD was determined according to a conventional protocol (Ceriello, Taboga et al. 2002; Sidhu, Newey et al. 2002; Arroyo-Espliguero, Mollichelli et al. 2003; Masci, Laclaustra et al. 2005). The right brachial artery was scanned continually throughout the test.

Pulse wave doppler was used to assess arterial flow at rest and a few sec after tourniquet deflation. Firstly, a baseline scan was performed for 2 min. During this time, the ECHO machine not only monitors the stable brachial artery endothelial walls, but also helps in measuring brachial artery diameter at regular intervals. Using pulse wave Doppler, the flow velocity and, subsequently, the blood flow could be calculated. At the same time, various cardiovascular parameters were monitored. Heart rate and regular BP checks were also monitored on a continuous basis. At the end of 2 min, the cuff placed on the distal right proximal forearm was inflated to a pressure of 200 mmHg almost instantly and left at that level of compression for 4 min 30 sec. During this transition, and also during the compression phase, both ultrasound parameters via ECHO and VIA parameters were continuously monitored on-line (Appendix II, Figure 2 b). Cardiovascular parameters were also monitored during this time. At 4 min 30 sec of compression, pulse wave doppler flow monitoring was started and at 5 min compression, the cuff pressure was released to zero. Pulse wave doppler was continued during the time when the cuff was released so that the velocity integral at the

time of release could be recorded and measured. After 30 sec of release, 2D ultrasound scanning was re-started to last until 5 min of recovery. Brachial artery dilatation (FMD) following reactive hyperaemia was measured at 50 sec following release of cuff pressure. The print out with the FMD result, along with all the cardiovascular data, was obtained immediately when the scanning ended. At the end of the last scan for the day, 250 µgrams of sublingual GTN was administered. Imaging was performed for another 5 min to determine endothelium independent vasodilatation (Figure 2.3.4.3 below).

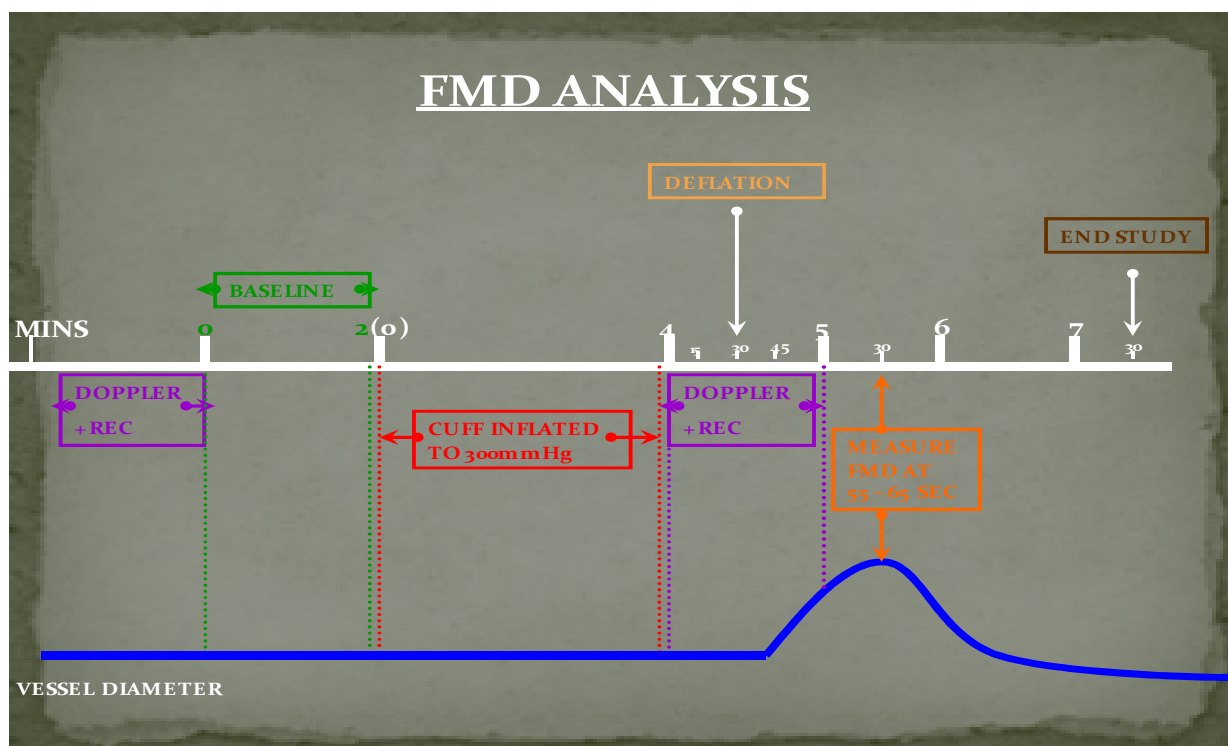


Figure 2.3.4.3 Protocol for Brachial artery scan using VIA software

The above picture shows the protocol used to scan the brachial artery, in the study. The white line is the time line showing 2 min of baseline scanning followed by 4 min 30 sec of compression. Finally, there is a further 2 min of scanning post release of compression. The FMD value was measured at 50 sec after release. The bottom blue line shows the artery diameter showing increased measurements at 50 sec point confirming the FMD percentage value.

At the end of the study a graph of diameter against time was immediately displayed. The FMD (defined by the percentage increase in mean diameter over a 10 sec period, 50 sec after tourniquet deflation) was automatically calculated by VIA from diastolic vessel diameters (Figure 2.3.4.4 and Figure 2.3.4.6 below).

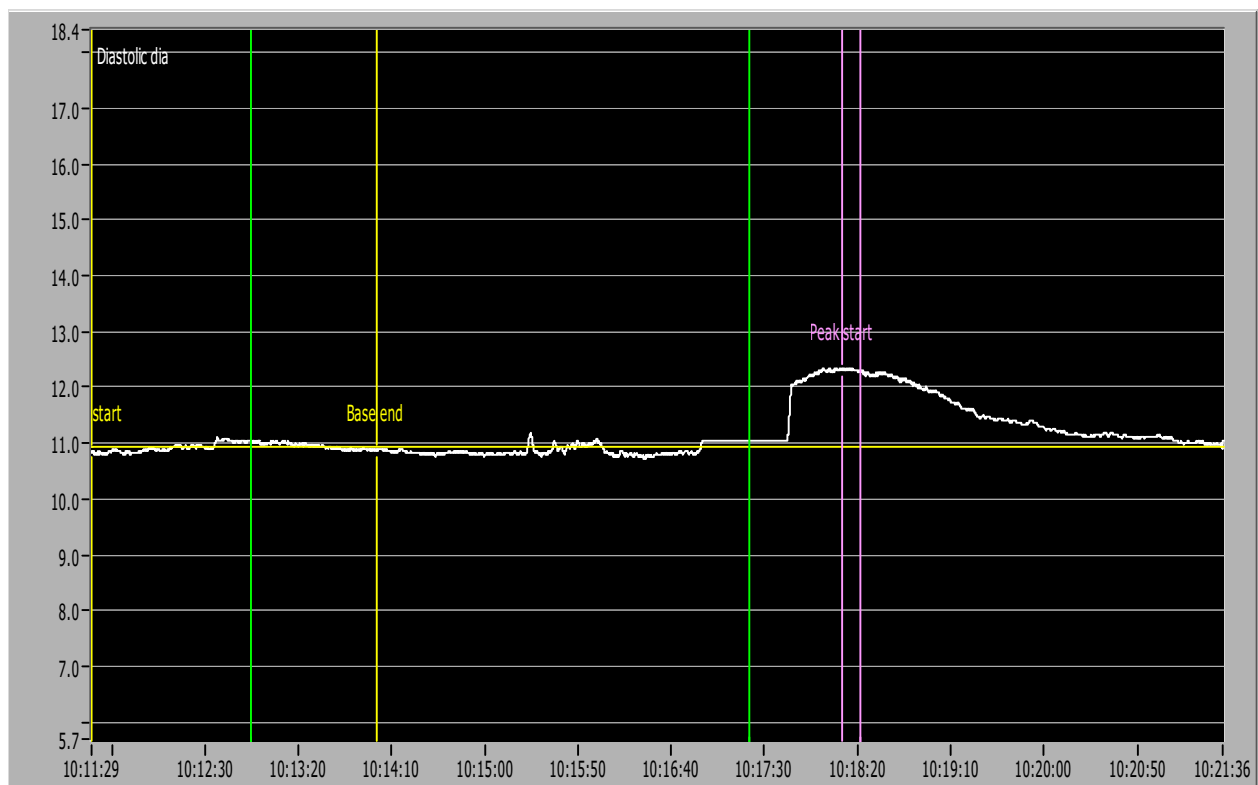


Figure 2.3.4.4 VIA software displaying real time diameter changes over time.

The Y axis displays arterial diameter in mm. The X axis displays time in minutes and seconds. The first and second green lines represent the time points at which the BP cuff inflated and then deflated, respectively. Purple lines represent the 50 sec FMD peak dilation point post deflation.

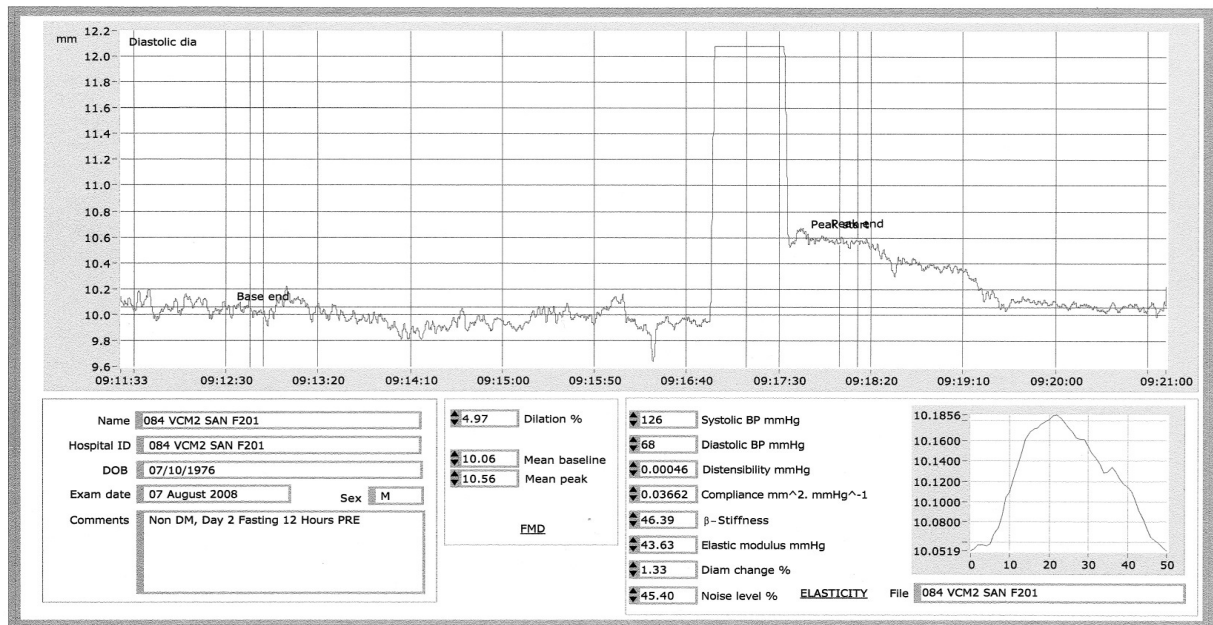


Figure 2.3.4.5 VIA software final reports on an FMD scan

The above figure shows the FMD % dilatation along with cardiovascular and elasticity parameters on a subject's scan.

The graph at the centre shows the dilatation at 50 sec in response to release of pressure on the arterial wall. The cardiac cycle is represented at the bottom right of the picture.

The endothelium independent dilatation was calculated similarly to the percentage increase in mean diameter 5 min after GTN administration. On-line image analysis and continuous display of vessel diameter allowed wall tracking to be optimized throughout the study, avoiding loss of data because of poor image quality. Using VIA software, the FMD was calculated and displayed within seconds of completing the study. It had already been shown that this new technique minimised the variability of measurement. The mean day to day variability of FMD measurements was 0.9 (0.5)% in subjects with a wide range of measured FMD and baseline arterial diameters in a previous study (Sidhu, Newey et al. 2002). Using a reference two dimensional image of the artery for comparison in repeat studies also ensured that the same section of artery was scanned every time each day and on subsequent days. The



magnitude peak FMD response could be influenced by any variability of occlusion cuff position, duration of occlusion, occlusion pressure and time of peak dilatation measurements (Pyke and Tschakovsky 2005). As noted above, all systems were standardized to minimize variability in the scanning procedures. Similar measurements to detect endothelial function were undertaken in the retinal vasculature to study microvascular function. The following procedure was used.

#### 2.3.5 Retinal Vessels Reactivity (RVR) Measurements

Retinal vessels facilitate for observations and measurements of microvascular changes in a direct and non-invasive method. This method is used by clinicians using an ophthalmoscope to identify changes in the vascular bed relating to diabetes, hypertension and other cardiovascular risk parameters but early functional vascular changes of T2DM and hypertension could occur without any identifiable or obvious lesions on traditional ophthalmoscopic examination (HICKAM and SIEKER 1960). Similarly, early functional changes in the retinal vascular bed may not be identifiable on current retinal screening programmes based on identifying structural abnormalities. Therefore, in order to identify these early functional vascular changes of vasosclerosis (when there is little or no change on ophthalmoscopic (HICKAM and SIEKER 1960) or retinal screening fundal photographs), RVA, with its ability to objectively measure vascular function, was utilized to measure microvascular function in this study.

RVR was measured using the retinal vessel analyzer (RVA, Imedos, GmbH). The fundamental components of this system consisted of a fundus camera (field of 50°; model FF 450; Zeiss, Jena, Germany), a video camera, a real-time monitor and a computer with image-analysis software for the accurate determination of retinal arterial and venous diameter.

Retinal vessel diameter was analysed in real time with a maximum frequency of 40 Hz, so that every second a maximum of 25 readings of vessel diameter could be obtained. For this purpose the fundus was imaged onto the charge-coupled device (CCD) (camera with an apparatus which was designed to convert optical brightness into electrical amplitude signals using a plurality of CCDs, and then reproduce the image using the electrical signals without time restriction) chip of the video camera (Appendix I, Figure 1 h). The consecutive fundal images were digitised with a frame grabber. Evaluation of the retinal venous and arterial diameter was performed on-line. These pictures could also be reviewed and re- analysed off-line, from the recorded videotapes (Seifertl and Vilser 2002).

#### 2.3.6 Mechanism of wall motion detection-Retinal Vessel Analyser (RVA)

The RVA system, as a method suitable for quantitative examination of retinal vessel function and changes of atherosclerosis, was first reported in 2004 in a clinical methodological study by Nagel (Nagel and Vilser 2004). The RVA measured the diameter of the retinal vessel. Each blood vessel has a specific transmittance profile due to the absorbance properties of haemoglobin. Measurement of retinal vessel diameter was based on adaptive algorithms that used those specific profiles. Whenever a specific vessel profile was recognised, the RVA system followed this vessel, as long as it appeared within the measurement window (Seifertl and Vilser 2002). The system corrected automatically for alterations in luminescence that were induced, for instance, by slight eye movements. If the requirements for the assessment of diameter were not fulfilled temporarily, as occurred during blinks, the system automatically stopped the measurement of diameter. As soon as an adequate fundus image was achieved again, measurement of diameter restarted automatically. An opto-electronic shutter controlled by a special program running on the RVA computer, inserted in the camera,

interrupted the observation light (530–600 nm, irradiance at the fundus  $\sim 1.96 \times 10^{-4} \text{ W/cm}^2$ ) over the entire 30° visual field of the retinal camera and produced a bright-to-dark contrast ratio of at least 25:1 (flicker provocation) (Seifertl and Vilser 2002). This created the flicker light by means of rectangular interruption of a green observation light of the RVA. Flicker provocation increased metabolic rate of the retinal vasculature and, in turn, acted as an indirect stimulant. The flicker stimulated retinal photoreceptors, which have a higher metabolic demand and influence vascular reactivity through neurovascular coupling. An ROI could be selected as a rectangle that included the particular vessel. Diameter was calculated along the arterial or venous segment, or both, that were located within the rectangle. As long as the vessels under study were within the selected rectangle during the eye movements, the system automatically corrected for the eye movements. This was again permitted by the adaptive nature of the diameter analysis software. In contrast with other procedures which had been proposed for the determination of retinal vessel size, this system measured diameters only in relative units (Polak, Dorner et al. 2000). RVA, as described previously and as used in this study, had been proven in a methodological study in 2004 (Nagel and Vilser 2004) as a method to observe light induced diameter changes.

### 2.3.7 Retinal Vessel Analyser (RVA) Protocol

All subjects were given topical 1 % Tropicamide applied to the right eye, at least 20-30 min before the actual scanning for pupillary dilatation. The subjects were seated comfortably for a few min and the stool height adjusted so that the subject was extremely stable and comfortable for the duration of the study. The left eye was covered to improve fixation of the right eye during imaging with the fundus camera. The image of the retina was adjusted on the screen of the real-time monitor after adjusting the camera to the dilated pupil, and a suitable

area in the retina was picked. The best area for scanning was picked based on the course of artery and vein, distance from the fundus and lack of branches. The superior or inferior quadrant of the temporal region was the usual area picked (Appendix I, Figure 1 i). BP and pulse-rate readings were taken before the start of the scan. The recordings were stored on a storable medium and could be evaluated off-line. After obtaining a clear fundus image with good contrast and no reflections, the scanning was started according to a fixed protocol. The fundus of the eye was examined under green light. After the baseline recording of 50 sec, where both artery and vein in the chosen segment were continuously monitored, three flicker periods of 20 sec each were applied, interrupted with 80 sec of still illumination. The flickers were created by interrupting the green light. The maximum vaso-motion, in response to flicker stimulation, was measured in two chosen segments of the major temporal inferior branch of the arteriole and venule. The point of maximum dilation was determined as the highest 1 sec mean vessel diameter during the period of 50 sec, after the beginning of the corresponding flicker stimulus (Gugleta, Zawinka et al. 2006). Three points of maximum dilation were obtained in all measuring sites (proximal and distal in arterioles and venules) and expressed as a percentage of the corresponding first baseline value. A graph was generated which described the measure of vessel dilatation (Appendix II, Figure 2 c and Figure 2 d).

#### 2.3.8 Arterio-venous Ratio (A/V Ratio) on RVA

Apart from the reactivity measurements of retinal vessels, the RVA contained software to measure other parameters including “vessel map basic” which enabled A/V ratios to be determined in line with the Atherosclerosis risk in communities study (ARIC) (Wong, Klein et al. 2001). The A/V ratio was a quantitative parameter to determine vascular risk. In

combination with an individual's medical history and an evaluation of micro-vascular fundus, this can result in a valuable vascular predictive tool. The result was obtained automatically once the set number of arteries and veins were identified on the fundal pictures (Appendix II, Figure 2 e).

## **2.4 Molecular Analysis**

### **2.4.1 Immediate bed-side plasma and serum isolation**

Serum and plasma from blood of participants was separated immediately after collection. Plasma was collected after spinning a column of blood in a mobile centrifuge (Capricorn Laboratory Equipment, model CEP 2000) at  $1500\times g$  for 15 min. Then the sample was immediately placed in a special container and then stored in a freezer straight away. Similarly serum from the blood was separated after collecting the blood in a collection tube which aided coagulation. Initially, up to 30 min of standing was allowed before spinning to aid coagulation. Once the blood was coagulated inside the collecting tube, the tube was placed in a centrifuge and spun at  $1500\times g$  for 15 min. This separated the serum at the top. Again the sample was immediately placed in a special container and then stored in a freezer straight away. Long term storage of serum, plasma and urine samples was carried out in 1.5 ml collection tubes and stored at  $-80^{\circ}\text{C}$  until further analysis. A detailed database was created in accordance with ethics procedures for long term storage of all the samples (Appendix II, Figure 2 f).

## 2.4.2 Multiplex Immunoassay

Serum endotoxin concentrations were assessed using a Chromogenic Limulus Amebocyte Lysate test (Cambex, New Jersey, USA), with a sensitivity of 0.1 EU/ml and an intra- and interassay CV of  $3.9 \pm 0.46$  and  $9.6 \pm 0.75$ , respectively. Our previous studies confirmed the specificity of the endotoxin assay (Baker, Harte et al. 2009). A multiplexed CVD Panel 1 immunoassay (Linco Research, Missouri, USA) was utilised to examine the circulating concentrations of the following inflammatory and CVD risk biomarkers: TNF- $\alpha$ , PAI-1 (tPAI-1, total) CRP, soluble intercellular adhesion molecule type-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), MMP-9, MPO, VEGF and soluble endothelial selectin (sE-Selectin). The CVD Panel 1 immunoassay had a sensitivity of 16-50,000 pg/ml for MMP-9, MPO and PAI-1 and further, a sensitivity of 80-250,000 pg/ml for sICAM-1, sVCAM-1 and sE-Selectin; with an intra- and interassay CV of 4.5-12.3% and 8.5-16.3%, respectively. Plasma glucose was measured using an automated glucose analyser (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured by a chemiluminescent assay (DPC, Los Angeles, USA) with a sensitivity of 14.3 pmol/l and intra- and interassay CV of 3.7% and 6.7%, respectively.

## 2.4.3 Protein Glycation and oxidation damage to LDL products

### 2.4.3.1 Isolation of LDL

LDL was isolated from the stored plasma samples. For rapid, same-day preparation of LDL, a self-generating gradient of iodixanol in a vertical rotor (S120VT) was used in a Sorvall MTX 150 micro ultra centrifuge (Hitachi). The density of plasma was increased to 12% using 60% iodixanol solution (OptiPrep; Axis-Shield). Plasma (0.9 ml) was layered under 0.9 ml of 9%

iodixanol in a 2ml ultracentrifuge tube (polyallomer, no. S302897A; Hitachi) and further void filled with 0.2ml nitrogen-purged PBS. The sample was centrifuged (501,000g, 16°C, 2.5 hr) with low acceleration and deceleration. LDL was washed with nitrogen-purged water (4ml × 3) over 100-kDa microspin filters (Amicon) to remove iodixanol. The LDL was stored at −20°C until further analysis. Sample handling was performed under subdued light. Protein concentration was measured by Bradford assay (Rabbani, Chittari et al. 2010). Lipid peroxidation of LDL was assessed by measuring thio-barbituric acid reactive substances (TBARS). TBARS were quantified by reference of the chromophoric response to a standard curve constructed from malondialdehyde tetramethyl acetal and expressed as malondialdehyde equivalents (Rabbani, Chittari et al. 2010). The purity of LDL was assessed by SDS-PAGE denaturing and agarose non-denaturing electrophoresis.

#### 2.4.3.2 *In vitro* modification of LDL

LDL was glycated minimally by methylglyoxal and glucose *in vitro* to assess the major glycation adducts formed. LDL glycated minimally by methylglyoxal (MG<sub>min</sub>-LDL) was prepared by incubation of methylglyoxal (200μmol/l) with LDL (4.2mg/ml) in PBS (0.4 mmol/l diethylenetriaminepentaacetic acid [DETAPAC], pH 7.4) at 37°C for 6 hr. The glycated and control LDL was washed extensively with argon-purged ice-cold water using ultra-spin filters (Amicon 100-kDa cutoff membrane from Millipore) at 4°C, and stored at 4°C under argon and used within 2 weeks. LDL glycated minimally by glucose (AGE<sub>min</sub>-LDL) was prepared by incubation of glucose (25mmol/l) with LDL (3mg/ml) in PBS (0.4mmol/l DETAPAC, pH 7.4) under argon (0.5ml) at 37°C for 7 days under sterile conditions. Control LDL was incubated without glucose. The glycated and control LDL was washed with argon-purged water ultrafiltrate at 4°C, and stored at 4°C under argon until

further analysis. Electrophoretic mobility of native and modified LDL on agarose gel electrophoresis was performed using a gel lipoprotein electrophoresis kit ( Helena kit) using barbital buffer, pH 8.6.

#### 2.4.3.3 Delipidation of LDL

An aliquot of LDL solution (20 $\mu$ l, 100 $\mu$ g) was transferred into a glass tube (50  $\times$  7.5mm) containing butylated hydroxytoluene in methanol (5 $\mu$ l, 2mg/ml), 20% trichloroacetic acid (100 $\mu$ l), and water (75 $\mu$ l), mixed well, left on ice for 10 min, and then centrifuged (10,000g, 15 min, 4°C). The supernatant was removed and the pellet washed with acetone (200 $\mu$ l) and diethyl ether (200 $\mu$ l) and dried under argon.

#### 2.4.3.4 Enzymatic digestion of apoB100

Delipidated protein was hydrolyzed exhaustively by modification of our collaborators previous work (Ahmed, Argirov et al. 2002). Protein was suspended in 100mmol/l potassium phosphate buffer, pH 7.4 (50 $\mu$ l). Pronase E (20 $\mu$ l, 2mg/ml in 10mmol/l KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) and 10 $\mu$ l penicillin (50units/ml) and streptomycin (50 $\mu$ g/ml) were added and the samples incubated at 37°C for 24 hr. Thereafter, 10 $\mu$ l each of prolidase and aminopeptidase solutions (2mg/ml in 10mmol/l KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) was added, and samples were incubated for a further 48 hr. All steps were performed under argon.



#### 2.4.3.5 Protein biomarker determination by LC-MS/MS

Fructosyl-lysine (FL), advanced glycation end products (AGE) and oxidation and nitration markers were determined in enzymatic hydrolysates of delipidated lipoprotein by stable isotopic dilution analysis LC-MS/MS (Thornalley, Battah et al. 2003; Rabbani, Chittari et al. 2010). This work was published in Diabetes journal in 2010 (Rabbani, Chittari et al. 2010).

### 2.5 Statistical Analysis

Statistical analyses for these studies were performed using SPSS 17 programme. Initially, Kolmogorov-Smirnov test was used to determine whether variables had a normal distribution (Appendix III, Table 2.5.1 and 2.5.2). These results showed that the variables were normally distributed. Paired student's t test was subsequently used to compare parameters before and after intervention within the same group. In the healthy subjects, this test was performed to check whether the differences in values obtained after OGTT were significantly different from the fasting levels in the variables tested. Further, the non-parametric test, Wilcoxon signed ranks test, was used to confirm data comparisons within the same groups. Finally, baseline data comparisons between different groups were performed using an unpaired student's t test. Statistical significance was defined as  $P < 0.05$ .

### 2.6 Validation of vascular tests

The results obtained from both scanning parameters were validated by the high levels of validity measurements recorded for each scan. On the FMD software, the obtained values were validated by the low levels of noise recorded for each scan. Accuracy of the results

depended on the scans generating less than 40% as noise levels for each study. This figure was reached whilst validating the software by the manufacturer of the software program (Sidhu, Newey et al. 2002). This study consistently recorded noise levels less than 40 % at each point of the study confirming the validity of the results obtained (Appendix III, 2.6.1 and 2.6.2). On the RVA software, the validity measurements of more than 40% (valid cycles) were considered as a reliable indicator for accuracy of the study results (Seifertl and Vilser 2002). This study recorded valid cycles higher than 50 % at each point confirming the validity of the RVR values obtained (Appendix III, 2.6.3 and 2.6.4).

## **2.7 Summary**

The above tests summarize the various techniques and tests performed in this thesis in different chapters. A summary sheet was created for each subject with collated results from the tests outlined in this chapter (Appendix II, Figure 2 g). A detailed summary of specific tests and methodology was shown in the relevant chapters.

## **Chapter 3**

**Impact of Acute Lipid ingestion on Circulating endotoxin**

**Levels with assessment of vascular function**

### 3.1 Introduction

The interrelationships between inflammation, insulin resistance and adipose tissue (AT) are critical in explaining increasing risk of developing T2DM (Laaksonen, Niskanen et al. 2004) (Tuttle, Davis-Gorman et al. 2004). It is known that low grade chronic systemic inflammation contributes to this risk, which appears altered by several factors such as increasing age, gender, ethnicity, genetics and dietary influences. However systemic inflammation appears to persist in T2DM subjects, despite medication, whilst the mechanisms and mediators of this continual inflammation appear less clear. Evidently, AT accumulation has a significant impact on disease risk in type 2 diabetes and inflammation possibly through hypoxia (Landini, Honka et al. 2015) but may merely act in response to systemic primary insults (Baker, Harte et al. 2009; Tabak, Brunner et al. 2009; Youssef-Elabd, McGee et al. 2011).

One potential cellular mechanism for increased inflammation may arise through activation of the innate immune system in human AT (Brun, Castagliuolo et al. 2007; Cani, Amar et al. 2007; Cani, Neyrinck et al. 2007; Creely, McTernan et al. 2007). Previous studies have shown that increased activation of the innate immune pathway may arise through excess circulating lipopolysaccharide (LPS) or endotoxin; which represents the outer cell wall membrane of gram-negative bacteria likely from the gut (Lin, Lee et al. 2000; Brun, Castagliuolo et al. 2007; Creely, McTernan et al. 2007; Dixon, Valsamakis et al. 2008; Al-Attas, Al-Daghri et al. 2009; Miller, McTernan et al. 2009). Our previous work has shown that endotoxin has an immediate impact on the innate immune pathway in human AT, acting via key receptors known as the toll like receptors (TLRs) which recognize antigens, such as the LPS component, to initiate an acute phase response to infection (Creely, McTernan et al. 2007; Baker, Harte et al. 2009). Stimulation of the TLRs leads to intracellular activation of NF $\kappa$ B, a key transcription factor in the inflammatory cascade that regulates the transcription

of numerous pro-inflammatory adipokines (Creely, McTernan et al. 2007; Youssef-Elabd, McGee et al. 2011). Therefore in vitro endotoxin may act as a mediator of inflammation through activation of NF $\kappa$ B, leading to a rapid response within AT that may be exacerbated by increased AT mass (Lin, Lee et al. 2000; Wellen and Hotamisligil 2005; Song, Kim et al. 2006; Kopp, Buechler et al. 2009; Shoelson and Goldfine 2009).

However clinical studies have also implicated gut derived endotoxin as a 'primary insult' to activate the inflammatory state, contributing to metabolic disease, with current cross-sectional data showing elevated systemic endotoxin levels in conditions of obesity, T2DM and coronary artery disease (Brun, Castagliuolo et al. 2007; Creely, McTernan et al. 2007; Dixon, Valsamakis et al. 2008; Al-Attas, Al-Daghri et al. 2009; Baker, Harte et al. 2009; Miller, McTernan et al. 2009). Within these studies circulating endotoxin is observed to be positively associated with waist circumference, waist-hip-ratio, insulin levels, inflammatory cytokines, as well as lipids including total cholesterol, triglycerides (TGs), LDL-cholesterol and negatively associated with HDL-cholesterol (Brun, Castagliuolo et al. 2007; Creely, McTernan et al. 2007; Dixon, Valsamakis et al. 2008; Al-Attas, Al-Daghri et al. 2009; Baker, Harte et al. 2009; Miller, McTernan et al. 2009). A recent review summarized endotoxin as a model to induce transient systemic inflammation even in healthy human subjects (Schedlowski, Engler et al. 2014).

Lipids present another important mediator of inflammation in CVD and T2DM, as human dietary studies have noted that cholesterol, trans-fatty acids (TFAs) and saturated fatty acids (SFAs) all increase the levels of atherogenic lipoproteins. Specifically, SFAs are noted to adversely affect vascular function whilst polyunsaturated fats (PUFAs; such as linoleic acid (18: 2n-6) and n-3 PUFA) appear beneficial (Hall 2009). As such the combined importance of dietary lipids and endotoxin in determining inflammatory and CVD risk may arise as

endotoxin has a strong affinity for chylomicrons (lipoproteins that transport dietary long chain SFAs through the gut wall) as endotoxin crosses the GI mucosa (Amar, Burcelin et al. 2008; Ghoshal, Witta et al. 2009; Moreno-Navarrete, Manco et al. 2010). As such, atherogenic and inflammatory risk may arise through a combination of dietary lipoprotein patterns and an increase in circulating endotoxin, exacerbated by feeding patterns (Hall 2009; Wyness 2009).

To address whether endotoxin might possess an associated vascular risk, previous work has examined whether endotoxin could represent a biomarker of atherosclerotic and cardiovascular risk. This prior cross sectional study (n=193) sought to address the impact of gender and ethnicity and relationship between lipoprotein patterns and circulating endotoxin. Age-adjusted endotoxin levels identified men to have a higher circulating endotoxin level than women ( $p=0.002$ ). Further that in examining ethnicity the study observed that endotoxin levels were highest in South Asians (13.3 EU/mL [95% CI 12.0 to 14.7]), who are at raised metabolic risk, followed by white Caucasians (12.5 (10.8 to 14.6) and lowest in individuals of African origin (10.1 EU/mL [9.1 to 11.1]).

Additionally that endotoxin levels were positively associated with waist circumference, waist-hip-ratio, total cholesterol, serum TGs and serum insulin levels and negatively associated with serum HDL-cholesterol; with circulating endotoxin appearing to represent a novel marker of atherosclerotic and CVD risk (Miller et al, 2009). Furthermore prior data has also highlighted that endotoxin levels fall with treatment with rosiglitazone, which also reduces blood pressure. Both inflammation and vascular injury impact on the risk of elevated blood pressure as noted in numerous previous studies (Wei et al, 2007, Bautista et al, 2005, Harte et al, 2005, Chae et al, 2001). However limited data has examined the potential impact of endotoxin to affect blood pressure (Wei et al, 2007), although it remains unclear whether this

could be viewed as a consequence of endotoxin levels or a marker of such an occurrence. In spite of this, previous data has assessed the prenatal exposure of endotoxin in Sprague-Dawley rats and the link with blood pressure. The Sprague-Dawley rats in this study were injected intraperitoneally with endotoxin/LPS (0.79mg/kg) between 8-12 days gestation. The findings from these studies noted that prenatal exposure to endotoxin led to an increase in blood pressure, independent of body weight, and, as such, may suggest that systemic levels of endotoxin in obese children and adolescents may contribute to its aetiology. This gives us encouraging evidence that endotoxin may affect the circulation and the vasculature. Examining this, post-prandially, through a fat loaded meal may have specific systemic and vascular impact which may be altered and further influenced due to different gut compromised metabolic states.

Therefore altering the lipid profile through dietary intervention may reduce endotoxin and the arising inflammatory and vascular response. Recent human studies have explored dietary effects of a high SFA, high carbohydrate meal on circulating endotoxin levels in healthy individuals. The findings revealed a substantial increase in circulating endotoxin, in subjects given a high fat meal, in conjunction with markers of inflammation - as noted from mononuclear blood cells (Cani, Amar et al. 2007; Ghanim, Abuaysheh et al. 2009).

Murine studies have also identified an association between endotoxin and insulin resistance, through infusion of endotoxin, with the same effect also noted by a high fat diet (Cani, Neyrinck et al. 2007); with insulin resistance and weight gain both impacting on gut permeability (Brun, Castagliuolo et al. 2007; Ghanim, Abuaysheh et al. 2009). In studies, to date, using either infused endotoxin as a bolus or derived from the gut due to dietary changes, both methods suggest endotoxin has the capacity to impact on the inflammatory pathways (Ghanim, Abuaysheh et al. 2009; Deopurkar, Ghanim et al. 2010).

However, it remains to be established whether diets in different metabolic states affect absorption of endotoxin. Also do such post-prandial circulating endotoxin levels correlate with systemic lipid changes post-prandially, being compounded in more insulin resistant states?

Therefore the study in this chapter sought to establish whether a high fat rich meal increased circulating endotoxin levels and whether this is altered in different metabolic disease states. Further whether a lipid meal may affect micro and macro-vascular reactivity in the different metabolic states and, finally, whether such an acute effect may, in part, be mediated through changes in circulating endotoxin levels as well as lipid profiles.



## 3.2 Patients and Methods

### 3.2.1 Study subjects

Non-obese controls (NOC, n=9), obese subjects (n=15), patients with impaired glucose tolerance (IGT; n=12) and T2DM (n=18) were included in the study. The details are shown in Table 3.2.3 below. Obesity and body weight was categorized in Table 3.2.1 as below according to The World health Organization (WHO).

Classification	BMI(kg/m)
Underweight	<18.50
Normal range	18.50 - 24.99
Overweight	$\geq 25.00$
Pre-obese	25.00 - 29.99
Obese	$\geq 30.00$
Obese class I	30.00 - 34.99
Obese class II	35.00 - 39.99
Obese class III	$\geq 40.00$

Table 3.2.1.1 The International Classification of adult underweight, overweight and obesity according to BMI. The World health Organization (WHO) – 2009

All subjects with T2DM were of South Asian origin except 1 subject, who was Afro-Caribbean in origin. Similarly all healthy controls were South Asian in origin except one who was Afro-Caribbean. South Asians have a significantly higher risk of developing Type 2 diabetes and its associated complications (Yudkin 1996{Chowdhury, 2006 #353; Chowdhury, Lasker et al. 2006). It is an emerging epidemic for the healthcare of people in the UK and globally. South Asians have a six-fold increase in their risk of developing type 2 diabetes compared to age matched Caucasian populations (Raleigh and Kiri 1997). South Asians present with diabetes at a younger age; they are therefore exposed to diabetes for a longer period than Caucasians, and this in turn leads to a greater risk of cardiovascular and microvascular complications (Barnett, Dixon et al. 2006).

All subjects were non-smokers. Screening blood tests were performed for both baseline measurements to qualify for the study, as well as to assess their glucose control. Routine blood tests included renal function, glucose, HbA1C and full cholesterol profile. All subjects had their height, weight, abdominal circumference and BMI measurements taken using standard equipment. Blood samples were taken either from the right or left ante-cubital vein in a sitting position. Blood pressure was checked with a blood pressure monitor on the left arm and rechecked after 2 minutes.

Finally a 12 Lead ECG was performed using the same machine for the duration of the study. Subjects included in the study had a normal resting ECG, normal renal function tests and blood pressure and no history of vascular disease. Detailed medical drug histories were taken on medications and those subjects on medication considered to lead to a change in inflammatory status were excluded, including the thiazolidinediones. Ethical approval was obtained from the Local Research Ethics Committee and all patients gave written consent.

	Normal Group	Obese Group	IGT Group	Type 2 Diabetes Group	p value
Parameters					
N	9	15	12	18	
Age (Years)	39.9 ± 11.8	43.8 ± 9.5	41.7 ± 11.3	45.4 ± 10.1	NS
Sex (M:F)	6:3	10:5	7:5	11:7	
Ethnicity	Asian: n=8 Afro-Caribbean: n=1	Asian n=15	Asian n=12	Asian: n=17 Afro-Caribbean: n=1	
#BMI (kg/m <sup>2</sup> )	24.9 ± 3.2	33.3 ± 2.5	32.0 ± 4.5	30.3 ± 4.5	Normal Vs obese: p<0.001; Normal Vs IGT: p=0.001; Normal Vs T2DM: p=0.003; IGT Vs Obese: NS; IGT Vs T2DM: NS; Obese Vs T2DM: p=0.019
Waist Circumference (cm)	86.9 ± 8.25	108.9 ± 17.9	106.4 ± 10.37	100.1 ± 10.2	Normal Vs obese: p=0.001; Normal Vs IGT: p<0.001; Normal Vs; T2DM:p=0.002; IGT Vs Obese: NS; IGT Vs T2DM: NS; Obese Vs T2DM: NS
#Estimated Glomerular Filtration Rate	105.62±19.71	91.07±17.40	105.92±19.77	92.28±24.36	NS

Table 3.2.1.2 The table presents the anthropometric data for the different cohorts. Data are presented as mean±SD. # denotes data with a non-Gaussian distribution that were transformed prior to statistical analysis, significance values are noted.

All research subjects (n=54) with and without T2DM were given a high-fat meal (standardized meal: 75g fat, 5g carbohydrate, 6g protein) following an over-night fast of 12-14 hours, as previously described (Ceriello, Assaloni et al. 2005). The cohort consisted of non-obese controls (ND): age: 39.9 (mean±SD) 11.8yr, BMI: 24.9 (mean±SD) 3.2Kg/m<sup>2</sup>; n=9; Obese: age: 43.8±9.5yr; BMI:33.3±2.5Kg/m<sup>2</sup>; n=15; impaired glucose tolerance (IGT): age: 41.7± 11.3yr; BMI: 32.0±4.5 Kg/m<sup>2</sup>; n=12; type 2 diabetes: age: 45.4±10.1yr; BMI:30.3±4.5Kg/m<sup>2</sup>; n=18. Blood samples were drawn at baseline (0hr), and post-prandially (1hr, 2hr, 3hr, 4hr,) and endotoxin and lipid levels measured.

### 3.2.2 In vivo Assessment of the Biochemical profile

On the assigned date, fasting blood samples were collected from participating subjects and lipid profiles and fasting plasma glucose determined using routine laboratory methods undertaken in the biochemistry laboratory at University Hospital Coventry and Warwickshire (UHCW). In brief, the routine blood tests included renal function, glucose, HbA1C and full cholesterol profile [Triglycerides(TG), high density lipoproteins (HDL) and low density lipoproteins (LDL)], as noted in Table 3.2.2.

Insulin measurements were performed by a solid phase enzyme amplified sensitivity multiplex immune assay (Millipore, Hertfordshire, UK), and glucose was measured by a glucose oxidase method (YSL 200 STAT plus). Homeostasis model assessment for Insulin resistance (HOMA-IR) was calculated for all patients using the HOMA formula:  $\text{HOMA-IR} = \text{Fasting insulin (mU/l)} \times \text{plasma glucose (mmol/L)} / 22.5$ .

Normal Group					
Parameters	Baseline	1-hour	2-hour	3-hour	4-hour
#TGs (mmol/L)	1.1 ± 0.07	1.3 ± 0.08*	1.8 ± 0.08***	2.2 ± 0.2**	2.4 ± 0.27**
Total Cholesterol (mmol/L)	4.9 ± 0.95	5.1 ± 0.85	5.1 ± 0.73	5.1 ± 0.94	5.2 ± 0.81
#LDL-Cholesterol (mmol/L)	3.2 ± 0.04	3.2 ± 0.03	3.1 ± 0.03	2.82 ± 0.04*	2.79 ± 0.04
#HDL-Cholesterol (mmol/L)	1.1 ± 0.004	1.1 ± 0.004	0.98 ± 0.03	1.06 ± 0.004	1.03 ± 0.008
#TNF-α (pg/ml)	8.0 ± 2.3	8.1 ± 2.2	7.4 ± 2.0	7.4 ± 1.8	8.0 ± 2.0
#Leptin (ng/ml)	16.8 ± 4.8	17.0 ± 5.3*	14.6 ± 4.0*	13.6 ± 3.5*	14.7 ± 4.4
#Endotoxin (EU/ml)	3.3 ± 0.15	4.0 ± 0.17*	4.32 ± 0.19	5.5 ± 0.64	6.3 ± 1.4
Obese Group					
Parameters	Baseline	1-hour	2-hour	3-hour	4-hour
#TGs (mmol/L)	1.6 ± 0.08	1.7 ± 0.08	2.3 ± 0.10 ***	2.7 ± 0.12***	3.0 ± 0.14***
Total Cholesterol (mmol/L)	5.3 ± 0.80	5.1 ± 0.67	5.1 ± 0.99	5.3 ± 1.0	5.2 ± 0.76
#LDL-Cholesterol (mmol/L)	3.56 ± 0.04	3.34 ± 0.02	3.0 ± 0.05***	3.0 ± 0.06***	2.9 ± 0.04***
#HDL-Cholesterol (mmol/L)	0.92 ± 0.01	0.90 ± 0.01	0.84 ± 0.01*	0.85 ± 0.008**	0.81 ± 0.01***
#TNF-α (pg/ml)	8.4 ± 1.3	8.4 ± 1.4	7.8 ± 1.6	7.5 ± 1.4*	7.5 ± 1.4*
#Leptin (ng/ml)	25.6 ± 3.1	23.3 ± 2.7***	20.7 ± 2.3	21.7 ± 2.6	21.4 ± 2.9***
#Endotoxin (EU/ml)	5.1 ± 0.94	4.2 ± 0.71	6.2 ± 0.49	7.8 ± 0.76**	7.7 ± 0.58**
IGT Group					
Parameters	Baseline	1-hour	2-hour	3-hour	4-hour

#TGs (mmol/L)	1.3 ± 0.03	1.5 ± 0.04***	1.9 ± 0.07 ***	2.5 ± 0.15 ***	2.5 ± 0.15 ***
Total Cholesterol (mmol/L)	4.9 ± 0.80	4.7 ± 0.74	4.8 ± 0.74	4.8 ± 0.77	4.8 ± 0.77
#LDL-Cholesterol (mmol/L)	3.24 ± 0.04	3.08 ± 0.03	2.9 ± 0.03**	2.6 ± 0.04**	2.66 ± 0.05**
#HDL-Cholesterol (mmol/L)	0.88 ± 0.008	0.86 ± 0.008	0.82 ± 0.006***	0.81 ± 0.008***	0.77 ± 0.01***
#TNF-α (pg/ml)	4.4 ± 2.1	4.3 ± 2.0	4.3 ± 2.0	4.1 ± 2.0	4.3 ± 2.1
#Leptin (ng/ml)	37.0 ± 2.7	32.6 ± 2.7***	32.0 ± 2.7*	31.1 ± 3.0**	33.0 ± 3.3**
#Endotoxin (EU/ml)	5.7 ± 0.10	5.8 ± 0.22	5.5 ± 1.0	7.4 ± 0.26 *	7.5 ± 0.20 *
T2DM Group					
Parameters	Baseline	1-hour	2-hour	3-hour	4-hour
#TGs (mmol/L)	1.4 ± 0.03	1.6 ± 0.05***	2.2 ± 0.08***	2.8 ± 0.12***	3.1 ± 0.13***
Total Cholesterol (mmol/L)	5.0 ± 1.0	4.8 ± 0.95*	4.8 ± 0.91	5.0 ± 1.0	4.9 ± 0.93
#LDL-Cholesterol (mmol/L)	3.17 ± 0.11	2.9 ± 0.07***	2.6 ± 0.10***	2.4 ± 0.16**	2.4 ± 0.14***
#HDL-Cholesterol (mmol/L)	1.0 ± 0.02	1.0 ± 0.02	0.94 ± 0.02***	0.92 ± 0.02***	0.86 ± 0.02***
#TNF-α (pg/ml)	8.6 ± 2.0	8.4 ± 2.0	8.4 ± 2.0	8.3 ± 1.8	8.1 ± 2.3
#Leptin (ng/ml)	18.1 ± 3.5	20.0 ± 5.0***	18.7 ± 3.7	18.2 ± 3.4	15.2 ± 3.3
#Endotoxin (EU/ml)	5.3 ± 0.54	5.5 ± 0.44	5.8 ± 0.34	9.8 ± 1.2**	14.2 ± 3.0**

Table 3.2.2 The table presents the variable data for the different cohorts over time, which were presented as mean±SD, \* denotes significant difference compared with baseline; # denotes data with a non-Gaussian distribution that were transformed prior to statistical analysis. Significance values \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

### 3.2.3 Analysis of circulating endotoxin levels

Serum endotoxin was analysed using a commercially available QCL-1000 LAL Endpoint Assay (Lonza, New Jersey, USA). The assay, and the values given by the manufacturer for intra-assay CV ( $3.9 \pm 0.46$ ) and inter-assay CV ( $9.6 \pm 0.75$ ), have been validated in our laboratory, as detailed previously (Creely, McTernan et al. 2007).

### 3.2.4 FMD Methodology

In brief, vascular reactivity measurements were evaluated using the flow-mediated vasodilatation (FMD) of the brachial artery. Vasodilatation responses of the brachial artery have been measured by ultrasound technique, the validity of which has been previously confirmed (M. C. Corretti 1995). Brachial artery FMD was determined according to a conventional protocol (Ceriello, Taboga et al. 2002; Sidhu, Newey et al. 2002; Arroyo-Espiguero, Mollicelli et al. 2003; Masci, Laclaustra et al. 2005).

The system used for this study allowed live on-line analysis with a single operator. The tracking and optimisation for most part was done automatically by the system software. In view of live analysis, high image acquisition rate was possible and results were obtained instantly. This obviated the need for retrospective analysis (Newey and Nassiri 2002).

### 3.2.5 RVA Methodology

Retinal vessel reactivity was measured using the retinal vessel analyzer (RVA, Imedos, GmbH). The fundamental components of this system consisted of a fundus camera (field of 50°; model FF 450; Zeiss, Jena, Germany), a video camera, a real-time monitor, and a computer with image-analysis software for the accurate determination of retinal arterial and venous diameter. Retinal vessel diameter was analyzed in real time with a maximum frequency of 40 Hz, so that every second a maximum of 25 readings of vessel diameter could be obtained. This method was detailed in chapter 2.

The consecutive fundus images were digitized with a frame grabber. Evaluation of the retinal venous and arterial diameter was performed on-line. These pictures could also be reviewed and re-analyzed off-line, from the recorded videotapes (Seifertl and Vilser 2002). Measurement of retinal vessel diameter was based on adaptive algorithms that used those specific profiles. Whenever a specific vessel profile was recognized, the RVA system followed this vessel as long as it appeared within the measurement window (Seifertl and Vilser 2002).



### 3.2.6 Statistical analysis

For assessment of the different variables, statistical analysis was undertaken using a paired student t-test, for intra-comparison of hourly time points versus baseline, and an unpaired student t-test for inter-comparisons. The threshold for significance was  $p < 0.05$ . Data in the text and figures are presented as mean $\pm$ SD or mean $\pm$ SEM. Correlations were determined with a Pearson correlation for continuous variables and Spearman correlation coefficient for non-continuous variables. Once a non-Gaussian distribution was logarithmically or square root transformed, statistical analysis was undertaken. All statistics were performed on SPSS version 17.0.

### 3.3 Results

#### 3.3.1 Baseline characteristics across Groups

Table 3.2.2 revealed the metabolic baseline and hour time point characteristics of the 4 groups analysed in this study. Age did not differ significantly between the groups whereas BMI was altered across the groups, with the non-obese control (ND) group possessing the lowest BMI ( $24.9 \pm 3.2 \text{ Kg/m}^2$ ; Table 3.2.1). The other 3 groups (Obese:  $33.3 \pm 2.5 \text{ Kg/m}^2$ \*\*\*↑; IGT:  $32.0 \pm 4.5 \text{ Kg/m}^2$ \*\*\*↑ and T2DM:  $30.3 \pm 4.5 \text{ Kg/m}^2$ \*\*↑ subjects; p value; \*\*\*p<0.001, \*\*p<0.01) differed significantly compared with ND subjects, whilst the T2DM group exhibited a significantly lower BMI than the obese cohort (p<0.05). Waist Circumference (wc) followed a similar pattern to the BMI data. ND subjects' wc was  $86.9 \pm 8.25 \text{ cm}$  versus Obese  $108.9 \pm 17.9 \text{ cm}$ \*\*\*↑; IGT:  $106.4 \pm 10.37 \text{ cm}$ \*\*\*↑ and T2DM:  $100.1 \pm 10.2 \text{ cm}$ \*\*↑ subjects (Table 3.2.1). The mean systolic blood pressures (SBP) of the groups were similar across all groups and did not change significantly over the 4hr duration, which was also noted for the diastolic blood pressures (DBP), (ND subjects: SBP:  $128.2 \pm 9.7 \text{ mmHg}$ , DBP:  $72.3 \pm 10.0 \text{ mmHg}$ ; Obese: SBP:  $128.2 \pm 10.7 \text{ mmHg}$ , DBP:  $73.4 \pm 7.8 \text{ mmHg}$ ; IGT: SBP:  $127.3 \pm 10.1 \text{ mmHg}$ , DBP:  $75.1 \pm 8.8 \text{ mmHg}$  and T2DM: SBP:  $129.1 \pm 10.1 \text{ mmHg}$ , DBP:  $74.5 \pm 6.9 \text{ mmHg}$ ).

As anticipated, the baseline ND group had significantly lower fasting plasma glucose levels compared with the T2DM group, whilst showing similar glucose levels to the obese and IGT cohorts (ND:  $4.7 \pm 0.69 \text{ mmol/L}$  Vs T2DM:  $8.1 \pm 1.8 \text{ mmol/L}$ \*\*\*↑; IGT:  $5.6 \pm 1.2 \text{ mmol/L}$ ; Obese :  $4.9 \pm 0.93 \text{ mmol/L}$ ; p values; \*\*\*p<0.001). Hb1Ac was similar in the obese and ND groups but significantly higher in the IGT and T2DM groups (ND:  $5.9 \pm 0.31\%$  Vs T2DM:  $7.5 \pm 1.12\%$ \*\*\*↑; IGT:  $6.3 \pm 0.47\%$ \*↑; Obese:  $5.9 \pm 0.49\%$ ; p values; \*\*\*p<0.001, \*p<0.05). Within each cohort, glucose levels did not significantly alter over the 4hr post-prandial time

period. The baseline lipid profile across the groups was comparable. Serum endotoxin levels were significantly lower in the baseline ND group compared with the IGT and T2DM groups (ND:  $3.3 \pm 0.15$  EU/ml, Vs Obese:  $5.1 \pm 0.94$  EU/ml $\uparrow$ ; IGT  $5.7 \pm 0.10$  EU/ml $^{**\uparrow}$ ; T2DM:  $5.3 \pm 0.54$  EU/ml $^{*\uparrow}$ ;  $^{**}p < 0.01$ ,  $^{*}p < 0.05$ ; Figure 5.3.2.1).

### 3.3.2 Post-prandial Change in Endotoxin levels over time in individual groups

Post-prandial exposure to a high-fat meal led to a significant rise in endotoxin levels in obese (baseline:  $5.1 \pm 0.94$  EU/ml; 1hr:  $4.2 \pm 0.71$  EU/ml; 2hr:  $6.2 \pm 0.49^{*}$  EU/ml; 3hr:  $7.8 \pm 0.76^{**}$  EU/ml; 4hr:  $7.7 \pm 0.58^{**}$  EU/ml;  $^{**}p < 0.01$ ,  $^{*}p < 0.05$  Figure 3.3.2.1); IGT (IGT: Baseline:  $5.7 \pm 0.10$  EU/ml; 1hr:  $5.8 \pm 0.22$  EU/ml; 2hr:  $5.5 \pm 1.0$  EU/ml; 3hr:  $7.4 \pm 0.26^{*}$  EU/ml; 4hr:  $7.5 \pm 0.20^{*}$  EU/ml;  $^{*}p < 0.05$  Figure 5.3.2.1) and T2DM subjects (Baseline:  $5.3 \pm 0.54$  EU/ml; 1hr:  $5.5 \pm 0.44$  EU/ml; 2hr:  $5.8 \pm 0.34$  EU/ml; 3hr:  $9.8 \pm 1.2^{**}$  EU/ml; 4hr:  $14.2 \pm 3.0^{**}$  EU/ml;  $^{**}p < 0.01$  Figure 3.3.2.1) over the 4hr time period. In the non-obese control (ND) group, whilst there was a rise in circulating endotoxin over the 4hr period this trend did not reach significance past 1hr (Figure 3.3.2.1). Fasting endotoxin levels showed a positive correlation with fasting TG levels in the whole cohort ( $r = 0.303$ ,  $p = 0.026$ ). Further examination of this relationship post-prandially identified the positive correlation strengthened over time, with the strongest relationship between endotoxin and TG noted at 2 and 3hr, respectively (2hr time point:  $r = 0.531$ ,  $p < 0.001$ ; 3hr time point:  $r = 0.498$ ,  $p < 0.001$ ) with a decline by 4hr post feeding ( $r = 0.434$ ,  $p = 0.001$ ). No further correlations between endotoxin and any other parameters were observed, and those noted were not influenced by age or gender.

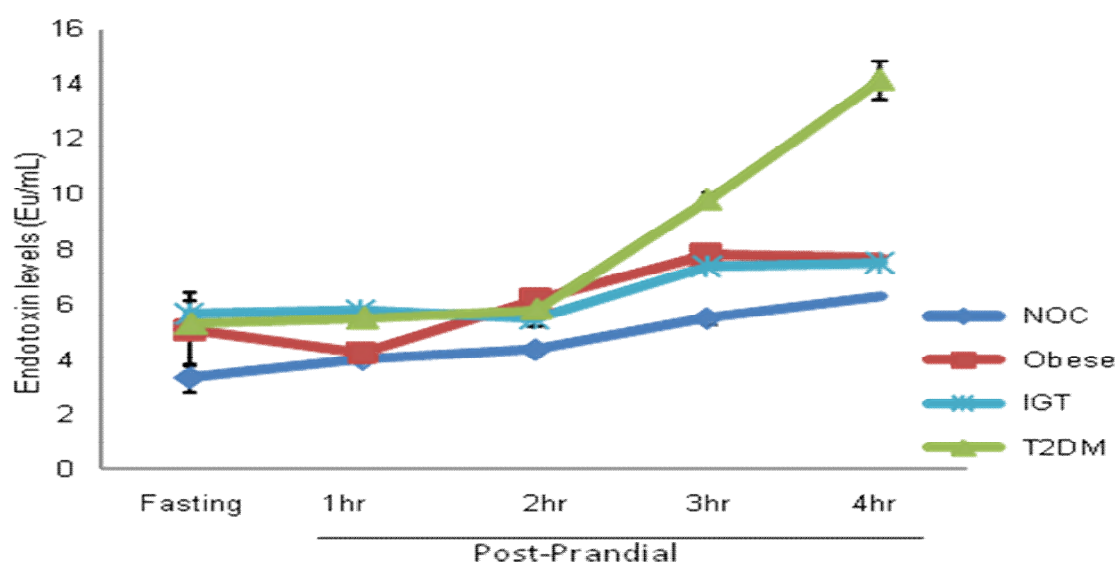


Figure 3.3.2.1 Endotoxin levels in NOC, IGT, obese and T2DM subjects

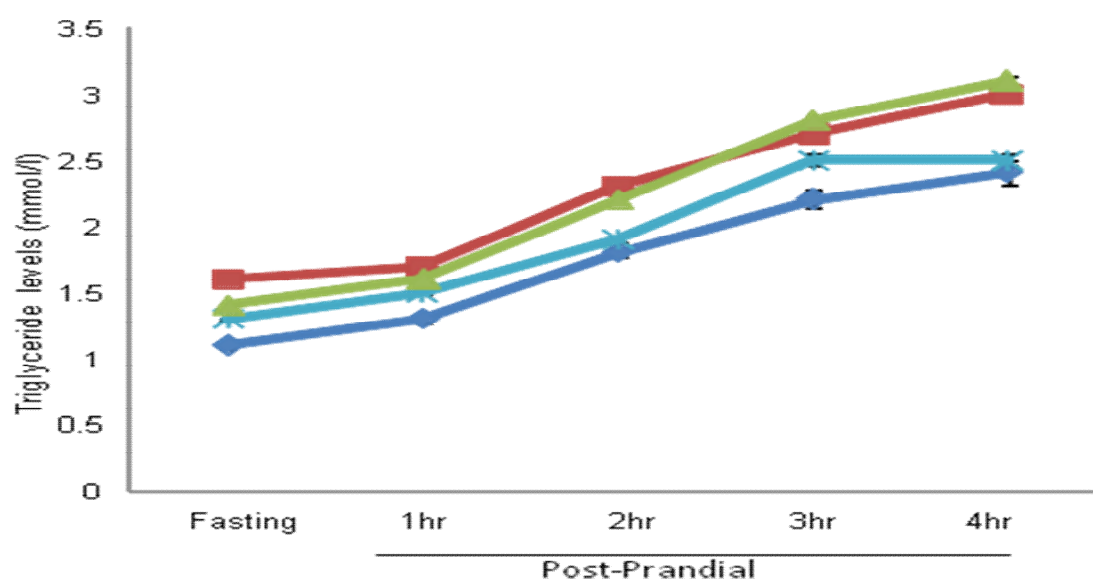


Figure 3.3.2.2

These figures show the changes in circulating endotoxin levels (3.3.2.1) and TG levels (3.3.2.2) in non-obese control (NOC), IGT, obese and T2DM subjects. Endotoxin and TG levels were measured at baseline and following a high SFA meal, at each hour post-prandially over a 4hr duration. Each point on the graph represents the mean value for each cohort ( $\pm$ SEM).

### 3.3.3 Post-prandial Change in endotoxin levels between groups

Fasting endotoxin levels were significantly higher in IGT and T2DM subjects compared with ND (72.7%\*\*and 60.6%\* increase, respectively; \*\* $p<0.01$ , \* $p<0.05$  Figure 3.3.3.2 & 3.3.3.3). However, during the post-prandial 4hr time period, the circulating endotoxin levels in both obese and IGT subjects diminished to approximately 20% higher than that of the ND (4hr, Figures 3.3.3.1 & 3.3.3.2), whilst circulating endotoxin levels were sustained at significantly higher levels in the T2DM subjects than the ND (4hr, Figure 3.3.3.3,  $p<0.05$ ).

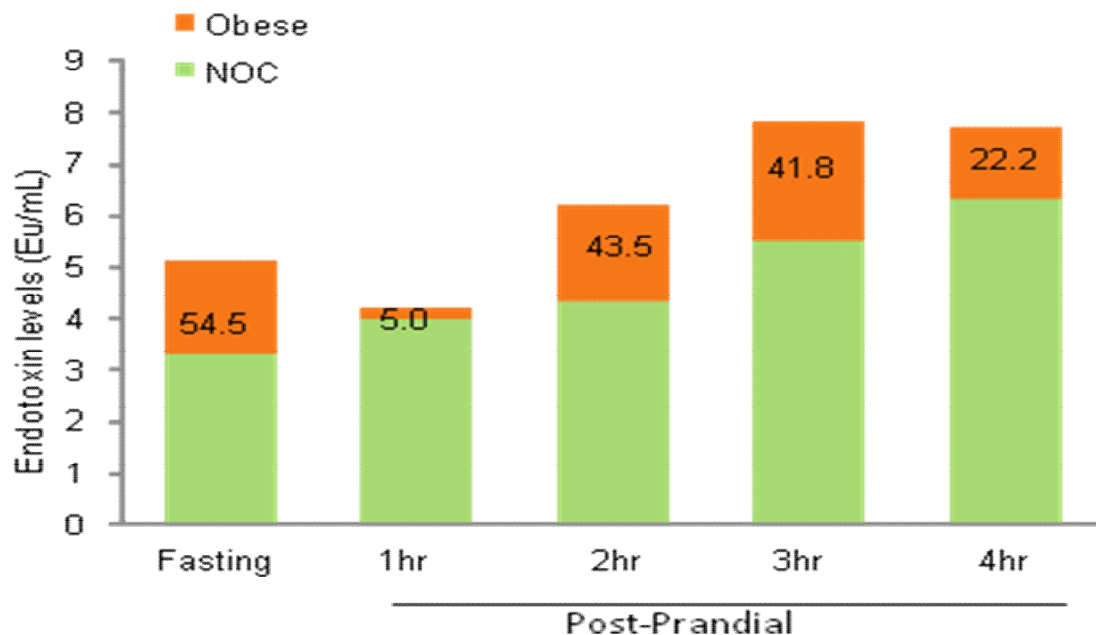


Figure 3.3.3.1 Endotoxin levels between the non-obese and obese subjects on fat stress

This figure shows the increase in endotoxin levels between the non-obese control (NOC) subjects and the obese subjects from baseline to 4hr post high fat meal. Endotoxin is measured in EU/mL and the percentage increase compared with NOC is also shown.

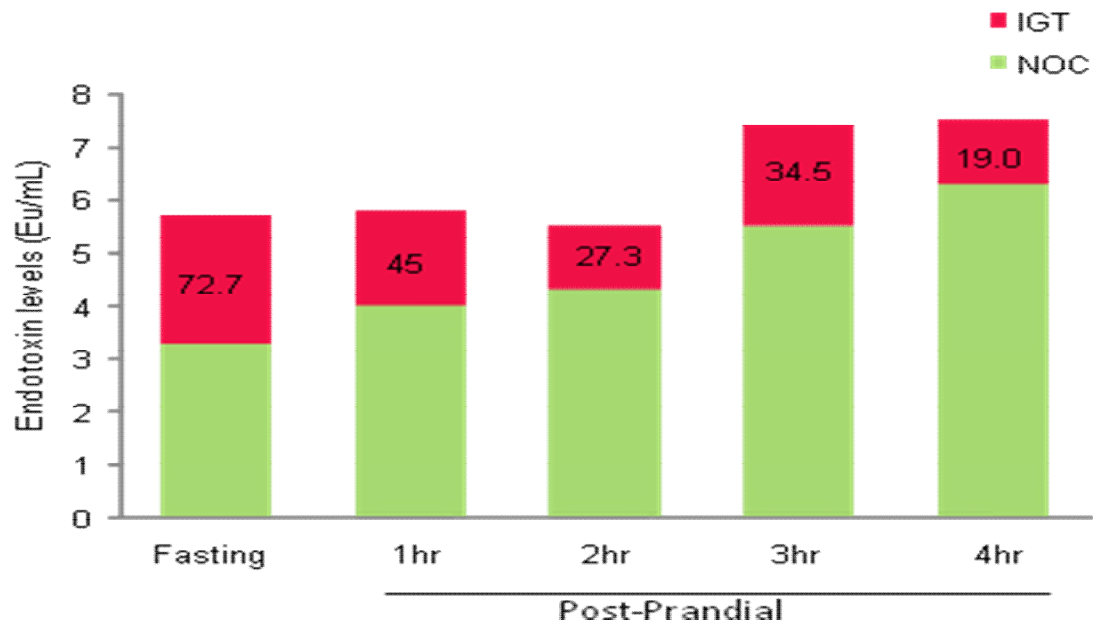


Figure 3.3.3.2 Endotoxin levels between the non-obese subjects (NOC) and IGT on fat stress

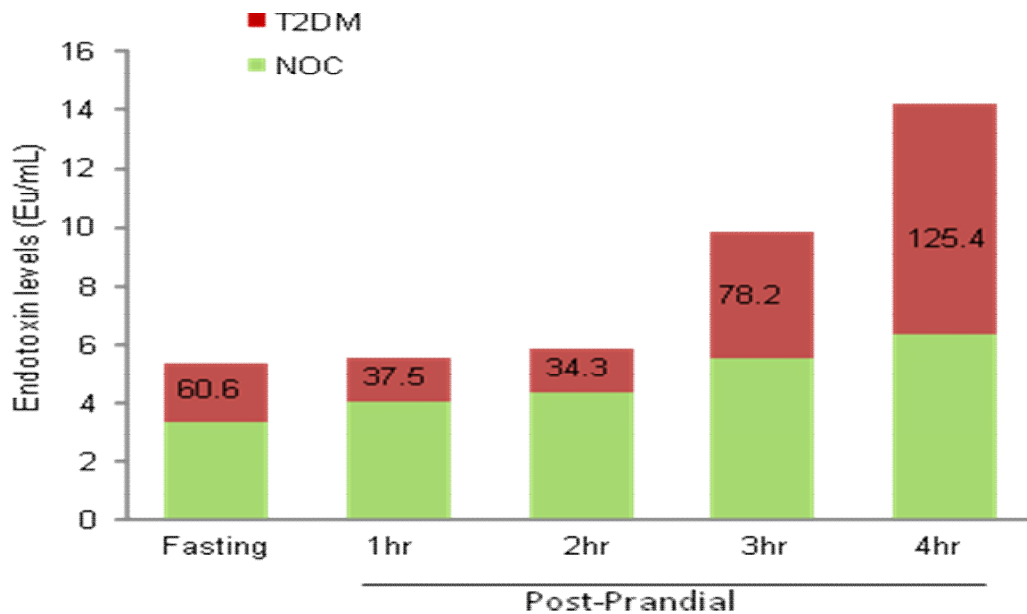


Figure 3.3.3.3 Endotoxin levels changes between NOC and T2DM subjects on fat stress

These figures show the increase in endotoxin levels between the non-obese controls (NOC) subjects, IGT (3.3.3.2) and T2DM (3.3.3.3) subjects from baseline to 4hr post high fat meal. Endotoxin is measured in EU/mL and the percentage increase compared to NOC is also shown.

### 3.3.4 Post-prandial Changes in lipids over time in individual groups

Post-prandial exposure to a high-fat meal led to a significant rise in TG levels in ND, IGT and T2DM subjects post 1hr ( $p<0.05$ ), whilst the obese subjects followed the same trend, TG levels were only significantly altered post 2hr feed ( $p<0.05$ ; Figure 3.3.2.2).

Total cholesterol remained relevantly unaltered over the 4hr period within all 4 groups (Table 3.3.1.1). In addition, no change was noted in LDL and HDL Cholesterol for the ND subjects over the 4hr period (Table 3.3.1.1). LDL and HDL in the other 3 groups did show significant individual group changes over time. For all metabolic states, LDL and HDL significantly changed (increased and reduced, respectively;  $p<0.05$ ; Table 3.3.1.1), whilst levels in ND subjects did not alter.

### 3.3.5 Post-prandial Changes in lipid levels between groups

Fasting total cholesterol, TG, LDL-Chol and HDL-Chol levels were comparable at baseline within the 4 groups and did not differ significantly throughout the 4hr duration (Figure 3.3.5.1; Table 3.3.1.1).

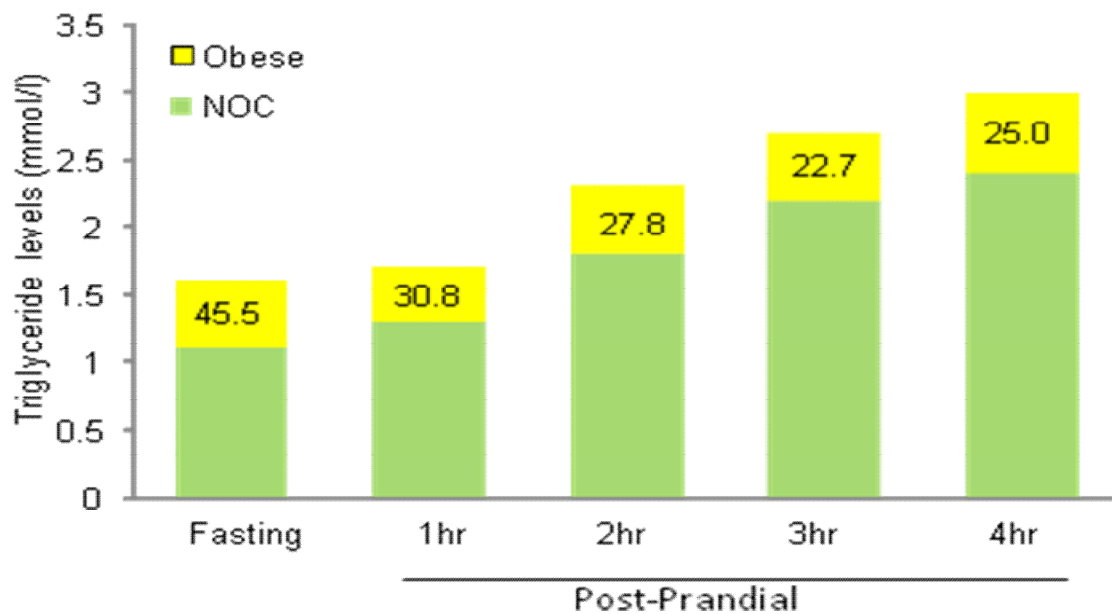


Figure 3.3.5.1 TG levels between the non-obese and obese subjects on fat stress

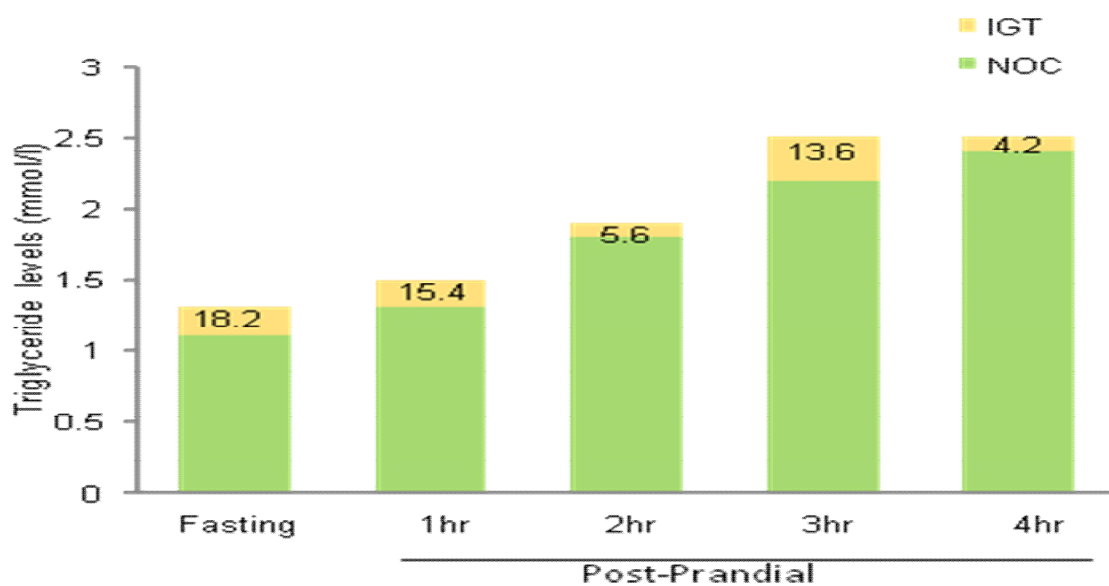


Figure 3.3.5.2 TG levels between the non-obese subjects and IGT on fat stress

These figures show the increase in TG levels between the non-obese control (NOC) subjects and the obese (3.3.5.1) and IGT (3.3.5.2) subjects from baseline to 4 hr post high fat meal. TG levels are measured in mmol/L and the percentage increase compared to NOC is also shown.



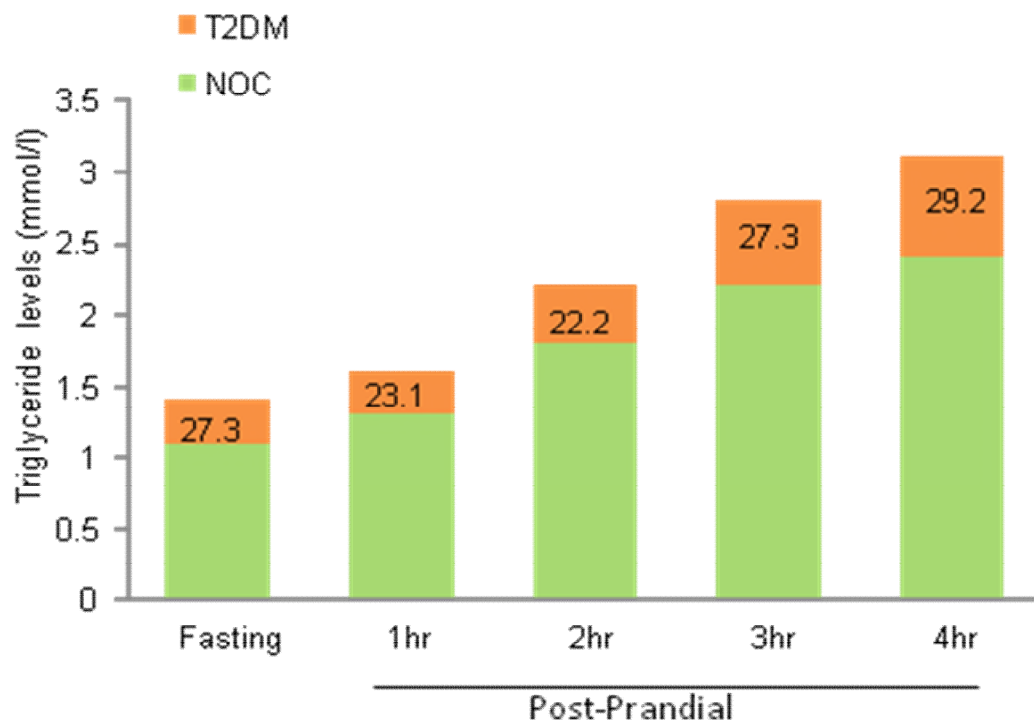


Figure 3.3.5.3 TG levels between the non-obese subjects and T2DM on fat stress

This figure reveals the increase in TG levels between the non-obese control (ND) subjects and T2DM subjects from baseline to 4hr post high fat meal. TG levels are measured in mmol/L and the percentage increase compared to ND is also shown.

### 3.3.6 Post-prandial Changes in FMD levels between groups

Post-prandial exposure to a high-fat meal led to a significant reduction in FMD % levels in all groups including ND, obese and T2DM subjects post 1hr ( $p < 0.05$ ) ( Tables 3.3.6.1, 3.3.6.2, 3.3.6.4), whilst the IGT subjects had similar significant depressed affect at 2 hours ( $p < 0.05$ ) (Table 3.3.6.3). These changes were mirrored by significant increase in endotoxin and triglyceride levels in all groups (Figure 3.3.2.1 and 3.3.2.2).

NOC		70 g Fat stress n=9			
FMD Brachial %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	4.00 $\pm$ 1.15	2.31 $\pm$ 0.87*	2.21 $\pm$ 0.67	1.19 $\pm$ 0.58*	2.37 $\pm$ 0.72*
Median $\pm$ Std. deviation	2.07 $\pm$ 3.64	1.13 $\pm$ 2.75	2.19 $\pm$ 1.90	0.67 $\pm$ 1.84	2.17 $\pm$ 2.30

Table 3.3.6.1 FMD of brachial artery in response to fat stress in ND subjects

The table presents the FMD data for the different NOC subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD,

\* denotes significant difference compared with baseline. Significance values \*p < 0.05.

T2DM		70 g Fat stress n=18			
FMD Brachial %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	2.30 $\pm$ 0.37	1.47 $\pm$ 0.37*	1.04 $\pm$ 0.64	1.04 $\pm$ 0.34*	2.42 $\pm$ 0.61
Median $\pm$ Std. deviation	2.29 $\pm$ 1.63	0.88 $\pm$ 1.62	0.59 $\pm$ 2.88	0.84 $\pm$ 1.59	1.44 $\pm$ 2.83

Table 3.3.6.2 FMD of brachial artery in response to fat stress in T2DM subjects

The table presents the FMD data for the different T2DM subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD,

\* denotes significant difference compared with baseline. Significance values \*p < 0.05.

IGT		70 g Fat stress n=12			
FMD Brachial %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	3.97 $\pm$ 1.05	2.70 $\pm$ 0.72	1.60* $\pm$ 0.88	3.45 $\pm$ 0.97	2.34 $\pm$ 0.96
Median $\pm$ Std. deviation	3.75 $\pm$ 3.33	2.63 $\pm$ 2.49	1.00 $\pm$ 3.05	2.97 $\pm$ 3.24	0.92 $\pm$ 3.34

Table 3.3.6.3 FMD of brachial artery in response to fat stress in IGT subjects

The table presents the FMD data for the different IGT subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD, \* denotes significant difference compared with baseline. Significance values \*p < 0.05.

Obese		70 g Fat stress n=15			
FMD Brachial %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	3.31 $\pm$ 0.67	1.47 $\pm$ 0.80*	1.77 $\pm$ 0.48*	1.85 $\pm$ 0.33	3.36 $\pm$ 0.52
Median $\pm$ Std. deviation	2.30 $\pm$ 2.52	1.76 $\pm$ 2.77	1.37 $\pm$ 1.87	1.69 $\pm$ 1.20	3.06 $\pm$ 2.03

Table 3.3.6.4 FMD of brachial artery in response to fat stress in obese subjects

The table presents the FMD data for the different obese subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD, \* denotes significant difference compared with baseline. Significance values \*p < 0.05.

Mean baseline (fasting) flow mediated dilatation (FMD) of brachial artery in NOC subjects (4.00 %) was significantly higher than the mean baseline levels noted in T2DM subjects

(2.30 %). Similarly, mean baseline level in NOC subjects was slightly higher than both obese subjects (3.31 %) and IGT group (3.97 %). After a high fat meal (70 grams equivalent), the mean FMD levels depressed significantly ( $P < 0.05$ ) in all groups as evidenced in tables 3.3.6.1-3.3.6.4.

These depressive effects seem to be more profound in T2DM compared with any other group even though it did not reach statistical significance (Table 3.3.6.5). Similarly, compared with healthy controls, IGT subjects had statistical significant variation in FMD levels by hour 3 on fat stress ( $p < 0.05$ - Table 3.3.6.5). By 4 hr after the fat stress, the FMD levels in all groups start to recover and reach the previous baseline values.

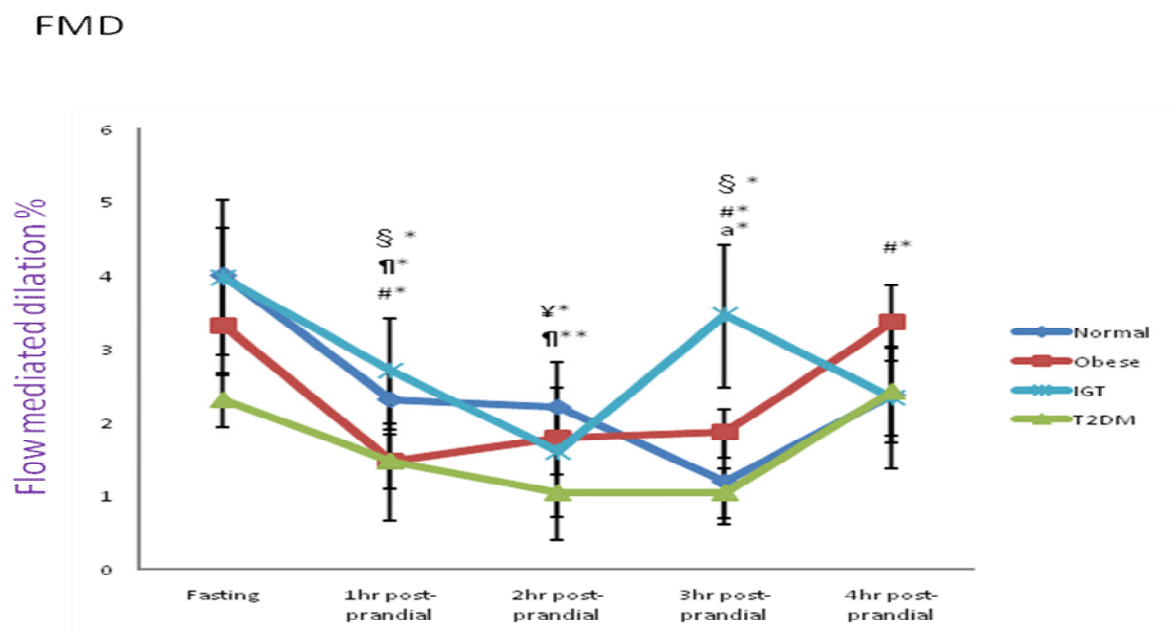


Figure 3.3.6.5 FMD changes between cohorts pre and post meal

Intra-group analysis, assessed flow mediated dilation (FMD) post-prandial percentage (%) changes; a\*=NOC (normal) vs IGT. #\*=Normal vs fasting; ¶=Obese vs fasting; ¥=IGT vs fasting; §=T2DM vs fasting. P-values are noted in graph as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### 3.3.7 Post-prandial Changes in RVA levels between groups

Mean baseline retinal vessel reactivity (RVR) of the retinal artery in NOC subjects (2.8%) was significantly higher than the mean baseline levels noted in T2DM subjects (1.64%) and slightly higher than obese subjects (2.66%). Following the high fat meal, the mean RVR levels were depressed in T2DM subjects (1.04%), but in NOC, obese and IGT subjects, RVR levels were higher than their respective baseline levels (Table 3.3.8.1- 3.3.8.4). At 2 hr point, this divergence in levels between these three groups (NOC, IGT and obese) to T2DM was significant altered ( $p < 0.05$ ; Table 3.3.8.5). By 4 hour post fat stress, the RVR levels appear to return back to their previous baseline values (Table 3.3.8.1- 3.3.8.4).

NOC		70 g Fat stress n=9			
RVA Retinal %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	2.80 $\pm$ 0.64	2.61 $\pm$ 0.41	3.12 $\pm$ 0.61	3.70 $\pm$ 0.57	3.06 $\pm$ 0.65
Median $\pm$ Std. deviation	3.00 $\pm$ 2.02	2.65 $\pm$ 1.31	3.80 $\pm$ 1.93	4.25 $\pm$ 1.63	3.50 $\pm$ 1.96

Table 3.3.7.1 RVA in response to fat stress in NOC subjects

The table presents the retinal vessel reactivity (RVR) data for the different non-obese control (NOC) subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD.

T2DM		70 g Fat stress n=18			
RVA Retinal %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	1.64 $\pm$ 0.51	1.70 $\pm$ 0.31	1.04 $\pm$ 0.33	1.46 $\pm$ 0.42	1.48 $\pm$ 0.43
Median $\pm$ Std. deviation	0.90 $\pm$ 2.31	1.45 $\pm$ 1.38	0.70 $\pm$ 1.51	1.15 $\pm$ 1.81	1.10 $\pm$ 1.91

Table 3.3.7.2 RVA in response to fat stress in T2DM subjects

The table presents the RVA data for the different T2DM subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD.

IGT		70 g Fat stress n=12			
RVR Retinal %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	3.68 $\pm$ 0.66	2.89 $\pm$ 0.62	3.75 $\pm$ 0.54	2.76 $\pm$ 0.44*	3.05 $\pm$ 0.58
Median $\pm$ Std. deviation	3.60 $\pm$ 2.19	3.10 $\pm$ 2.08	3.40 $\pm$ 1.89	2.65 $\pm$ 1.53	2.95 $\pm$ 2.03

Table 3.3.7.3 RVA of Retinal artery in response to fat stress in IGT subjects

The table presents the RVA data for the different IGT subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD, \* denotes significant difference compared with baseline. Significance values \*p < 0.05.

Obese		70 g Fat stress n=15			
RVR Retinal %	Fasting	1 hr post	2 hr post	3 hr post	4 hr post
Mean $\pm$ std. Error	2.66 $\pm$ 0.43	2.76 $\pm$ 0.60	2.70 $\pm$ 0.52	3.03 $\pm$ 0.59*	2.76 $\pm$ 0.63
Median $\pm$ Std. deviation	2.85 $\pm$ 1.62	2.20 $\pm$ 2.17	3.00 $\pm$ 1.97	3.30 $\pm$ 2.12	3.10 $\pm$ 2.44

Table 3.3.7.4 RVA of Retinal artery in response to fat stress in obese subjects

The table presents the RVA data for the different obese subjects over time (baseline and post meal), which are presented as mean $\pm$ SE and mean $\pm$ SD, \* denotes significant difference compared with baseline. Significance values \*p < 0.05.

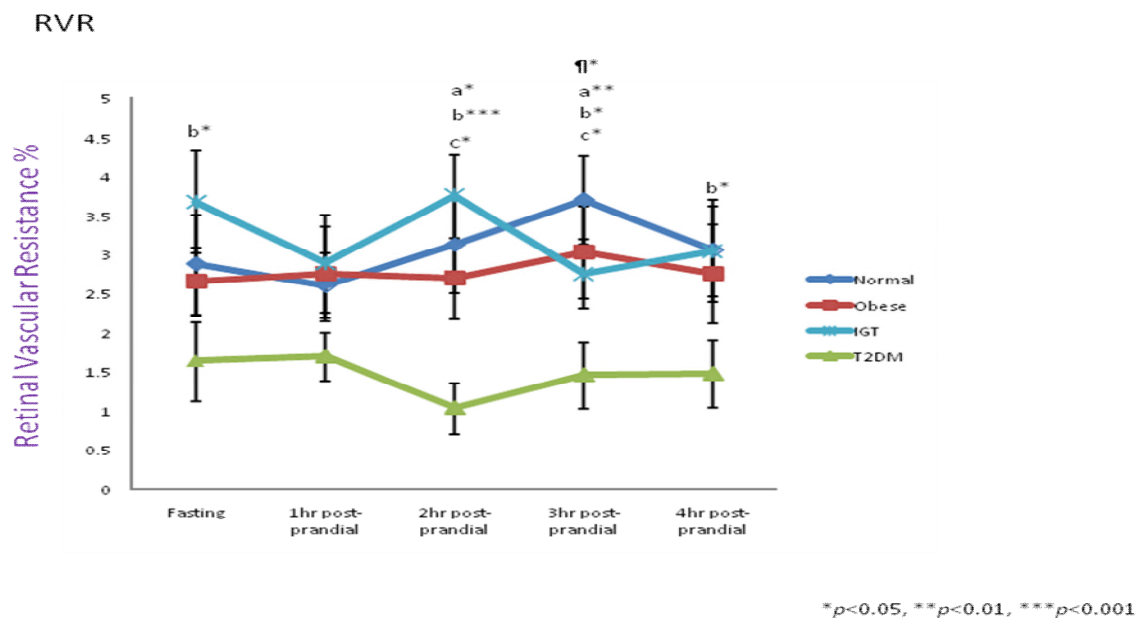


Figure 3.3.7.5 RVR changes between cohorts pre and post meal

Intra-group analysis, assessed retinal vessel reactivity (RVR) post-prandial percentage (%) changes;. a=T2DM vs NOC (normal); b=T2DM vs IGT; c=T2DM vs obese. #=normal vs fasting; ¶=Obese vs fasting; ¥=IGT vs fasting; §=T2DM vs fasting. P-values are noted in graph as follows: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

### 3.3.8 Correlation between endotoxin levels and vascular function

There was no correlation noted between changes in endotoxin and either FMD or RVR changes pre or post meal as detailed in Table 3.3.8.1.

Time point	Pearson correlation coefficient for log endotoxin Vs FMD	P value (2-tailed significance)	Pearson correlation coefficient for log endotoxin Vs RVA	P value (2-tailed significance)
Fasting	-0.060	(0.681)	-0.045	(0.025)
1 hr post prandial	-0.059	(0.687)	0.025	(0.045)
2 hr post prandial	-0.190	(0.187)	0.069	(0.076)
3 hr post prandial	-0.026	(0.858)	0.125	(0.089)
4 hr post prandial	-0.160	(0.253)	0.126	(0.002)

Table 3.3.8.1 Correlation between endotoxin and RVR or FMD. This table presents the Pearson correlation between log endotoxin and FMD and RVA, respectively at fasting and 1-4hrs post-prandial.



### 3.3.9 The effect of cholesterol levels on vascular reactivity

Analysis of FMD versus cholesterol (log transformed) identified a significant negative correlation at fasting and 1hr post-prandial, although absent at 2hr and 3hr. However, the strongest relationship between FMD and cholesterol was observed at 4hr, (4hr time point:  $r = -0.411$ ,  $p < 0.01$ ; Table 3.3.9.1). No further correlations between FMD and any other parameters were observed.

Time point	Pearson correlation coefficient for log cholesterol Vs FMD	P value (2-tailed significance)
Fasting	-0.320*	0.025
1 hr post-prandial	-0.288*	0.045
2 hr post-prandial	-0.253	0.076
3 hr post-prandial	-0.243	0.089
4 hr post-prandial	-0.411**	0.002

Table 3.3.9.1 The table presents the Pearson correlation between log cholesterol Vs FMD in the whole cohort at fasting and 1-4hrs post-prandial. \*\*Correlation is significant at the 0.01 level (2-tailed). \*Correlation is significant at the 0.05 level (2-tailed).

Analysis of FMD versus cholesterol (log transformed) identified a significant negative correlation at fasting and 1hr post-prandial, although absent at 2hr and 3hr. However, the strongest relationship between FMD and cholesterol was observed at 4hr, (4hr time point:  $r=-0.411$ ,  $p<0.01$ ; Table 3.3.9.1). No further correlations between FMD and any other parameters were observed.

### **3.4 Discussion**

This was the first study to examine the comparative and differential changes in circulating endotoxin following a SFA (saturated fatty acid) meal from subjects with and without T2DM, obesity or impaired glucose tolerance (IGT) status. The novel data highlights that a SFA meal increases circulating endotoxin levels in all subjects irrespective of their metabolic status, although circulating endotoxin shows dramatic post-prandial changes in the high metabolic risk groups. More specific comparative analysis of ND subjects versus subjects with T2DM at 4hr post-prandial, identified that the latter had a mean endotoxin level 125.4% higher than ND subjects. Cumulative data derived from the fasting state and the SFA post-prandial state indicate that T2DM subjects are subjected to 336% more circulating endotoxin than ND subjects over the 4hr duration. In comparison with other metabolic states, the obese and IGT subjects were still subjected to 167% and 198.5% more circulating endotoxin than ND subjects. As such, endotoxin, which is considered a potential mediator of chronic low grade inflammation, is considerably higher in the state of T2DM, with implications for a continual inflammatory state, as other papers have observed (Al-Attas, Al-Daghri et al. 2009; Ghanim, Abuaysheh et al. 2009; Miller, McTernan et al. 2009; Deopurkar, Ghanim et al. 2010).

Our previous studies have shown significant associations between SFA meal and adhesion molecules, particularly intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin (Ceriello, Quagliaro et al. 2004), all leading to endothelial dysfunction and association with CVD (Ceriello, Quagliaro et al. 2004). More recent study from Spain appear to confirm ED due to high fat meal (Garcia-Prieto, Hernandez-Nuno et al. 2015). Interest has been generated in inflammatory mediation from factors such as endotoxin, visfatin, TNF- $\alpha$  and Leptin (Eggesbo, Hjermann et al. 1994; Cani, Amar et al. 2007; Ghanim, Abuaysheh et al. 2009; Deopurkar, Ghanim et al. 2010; Lassenius, Pietilainen et al. 2011; Maitra, Gan et al. 2011; Agueda, Lasa et al. 2012; Tian, Chang et al. 2014; Pina, Genre et al. 2015; Xiao, Zou et al. 2015). Elevated levels of these factors have been reported in T2DM patients leading to new insight into endothelial dysfunction and cardiovascular risk.

Postprandial hypertriglyceridaemia and hyperglycaemia are considered risk factors for CVD, and evidence suggests that postprandial hypertriglyceridaemia and hyperglycaemia may induce an increase in circulating adhesion molecules (Ceriello, Quagliaro et al. 2004). Previous studies have revealed an independent and cumulative effect of postprandial hypertriglyceridaemia and hyperglycaemia on ICAM-1, VCAM-1 and E-selectin plasma levels, suggesting markers of inflammation as a common mediator of such effects (Ceriello, Quagliaro et al. 2004; Ceriello, Assaloni et al. 2005).

This chapter, therefore, looked at the effects of SFA/high fat meal on markers of inflammation, including endotoxin and TNF- $\alpha$ . Whilst our previous studies have shown significant associations in the fasted state between circulating endotoxin, lipoprotein patterns and anthropometric data, (Brun, Castagliuolo et al. 2007; Creely, McTernan et al. 2007; Dixon, Valsamakis et al. 2008; Al-Attas, Al-Daghri et al. 2009; Baker, Harte et al. 2009;

Miller, McTernan et al. 2009), these current studies sought to establish whether endotoxin acutely changes post-prandially, and whether this is altered by differing metabolic states. By undertaking this, our current studies have highlighted subtle but significant differences in how endotoxin levels change in the post-prandial period. Following the SFA meal, the NOC endotoxin levels rose over the 4hr duration but circulating levels did not increase significantly. In contrast, in the obese and IGT groups there was a significant rise in endotoxin, which appeared to plateau by 4hr. However, at the 4hr time point, both the IGT and obese groups' endotoxin levels were much lower than the T2DM subjects, as the levels of endotoxin in the T2DM subjects appeared to be still rising post 4hr SFA meal. Circulating endotoxin in the T2DM group, post 4hr, did not appear to 'normalize', which suggests the cumulative exposure to endotoxin after a high SFA meal is disproportionately high compared across any other group. This is in accordance with our previous data (Ceriello, Taboga et al. 2002; Ceriello, Assaloni et al. 2005) that high fat/SFA meal appears to lead to a significant rise in inflammatory markers as well as deterioration in endothelial function.

Furthermore, in the T2DM subjects the rising endotoxin levels may be further compounded by re-feeding. These data appear to indicate that a person eating 3 high SFA meals each day may encounter endotoxin levels that remain perpetually high, as re-feeding may increase the levels. As such, fasted endotoxin data, whilst important, may appear to miss the daily variation, as feeding data appears to show. The type of meal is clearly important, as previous studies highlight that dietary changes alter circulating endotoxin and influence inflammation, even in healthy subjects (Cani, Amar et al. 2007; Ghanim, Abuaysheh et al. 2009; Schedlowski, Engler et al. 2014). In addition, recent studies have reported that the simultaneous ingestion of certain 'healthy' food groups with saturated fat can negate an increase in circulating endotoxin and the customary inflammatory response (Deopurkar, Ghanim et al. 2010). As it is acknowledged that obese and T2DM subjects tend to eat high

SFA without correspondingly high levels of fruit or healthy foods, this diet would clearly impact on their endotoxin and inflammatory status (Arnold, Mann et al. 1997; Ghanim, Abuaysheh et al. 2009; Vitolins, Anderson et al. 2009). Therefore, a high SFA intake could represent a continual inflammatory insult for T2DM subjects, daily.

In the obese and IGT groups, the post-prandial 4hr endotoxin levels appear to plateau, whilst still remaining high compared with NOC subjects. Subsequently, another SFA meal may compound the circulating endotoxin levels further within the obese and IGT groups; therefore the type and frequency of meals may significantly impact on the metabolic risk. In addition to the type of meal, the food intake frequency is also relevant - although currently there are very few studies examining the importance of this.

Previous studies indicate no difference between a diet based on 3 meals a day or a diet comprising of smaller meals and snacks, with regard to the long-term effects on glucose, lipid, or insulin responses - although the unknown acute post-prandial effects on the inflammatory status may have a more profound long-term impact (Beebe, Van Cauter et al. 1990; Arnold, Mann et al. 1997). In addition, other studies have often stressed the division of food intake should be based on individual preference, with no clear recommendations on pattern of food intake. Within T2DM clinics, the recommendation for patients currently is to consume 5 smaller meals per day. This may arise to reduce the potentially overwhelming orexigenic effects patients might experience with only 3 meals a day, as well as the potential spikes in insulin, although the data do not necessarily give clear insight into these benefits (Beebe, Van Cauter et al. 1990; Arnold, Mann et al. 1997). In any of these studies, compliance of subjects to advice and meal frequencies is difficult to verify.

Besides the role of endotoxin on inflammatory status, previous studies have shown that increasing endotoxin levels appear to reduce the reactivity of retinal microvasculature, even

in healthy subjects (Kolodjaschna, Berisha et al. 2008). Such studies have shown that Intravenous administration of endotoxin leads to reduced measurements on the RVA similar to the findings in this study (Kolodjaschna, Berisha et al. 2008); (Blum, Bachmann et al. 1997). Similarly fat intake, even in healthy people, leads to a higher endotoxin level (Erridge, Attina et al. 2007; Grunfeld and Feingold 2009).

In this current study, circulating endotoxin appears to have risen in line with expectations, as a result of the high fat meal. Similarly, these high levels of endotoxin in the bloodstream resulted in a depressive effect on both macrovascular and microvascular function, as noted in previous studies. Subjects with T2DM, with the highest circulating endotoxin compared with other groups, showed a depression in vascular reactivity for a prolonged period, both at the macro and microvascular levels. Animal studies have already revealed that chronic endotoxaemia results in a metabolic syndrome ranging from obesity, IGT and T2DM (Turnbaugh, Ley et al. 2006; Grunfeld and Feingold 2009). This chapter may support this theory but has been unable to provide direct significant associations between either changes in FMD or RVR data and endotoxin levels. Such a lack of correlation may have arisen due to the size of the cohort, as well as the differential responses within each cohort, which may have produced a wide variation in response to the fat meal.

However, from this study it was demonstrated that SFA meal leads to a statistically significant increase in lipid levels, particularly triglycerides, which correlated with depressed endothelial function at the macrovascular level. This study also demonstrated that rising levels of lipids in all groups followed a similar pattern to that of increasing endotoxin levels. Therefore, we might be able to deduce that increasing endotoxin levels, as part of a SFA meal, appear to show a trend with a simultaneous fall in endothelial function at the macrovascular level, in all groups. Further studies would be required, however, to assess

these trends in a larger cohort in order to determine a significant effect, as previous studies have noted (Kolodjaschna, Berisha et al. 2008)

Based on these current studies, more frequent saturated fat exposure may exacerbate endotoxin, inflammation and depress vascular reactivity, further. Also, smaller more frequent meals have the potential to allow endotoxin to spike several times a day, thus activating the innate immune system within adipose tissue without desensitization (Creely, McTernan et al. 2007; Ghanim, Abuaysheh et al. 2009; Deopurkar, Ghanim et al. 2010; Youssef-Elabd, McGee et al. 2011). As such, the resulting downstream production of diabetogenic cytokines would be in continuous production, as previous in vivo and in vitro studies have demonstrated (Creely, McTernan et al. 2007; Ghanim, Abuaysheh et al. 2009; Deopurkar, Ghanim et al. 2010; Youssef-Elabd, McGee et al. 2011).

The TG levels did not differ significantly across the 4 groups of subjects at any of the time points; however the TG levels did increase from baseline to 4hr within each group, in a similar pattern to circulating endotoxin, but most significantly in the metabolic risk subjects (obese, IGT and T2DM), whilst also demonstrating an association with endotoxin, over the 4hr period (Creely, McTernan et al. 2007; Miller, McTernan et al. 2009). The 3 different metabolic states showed significantly higher fasting TG levels than NOC subjects, which post-prandially became further exacerbated in the obese and T2DM subjects.

Unsurprisingly, post-prandial TG levels increased in a similar pattern to circulating endotoxin, whilst also demonstrating an association, over the 4hr period (Creely, McTernan et al. 2007; Miller, McTernan et al. 2009). The 3 different metabolic states showed no significant differences in TG levels compared with levels in NOC subjects. However, the significant correlation between fasting TG and endotoxin levels confirms previous studies in which an association between these two metabolic parameters had been observed (Creely,

McTernan et al. 2007; Miller, McTernan et al. 2009). Our data indicated that the association between TGs and circulating endotoxin became stronger in the post-prandial state each hour over the 4hr duration, substantiating previous evidence that lipids mediate the transfer of endotoxin from the GI tract into the circulation (Brun, Castagliuolo et al. 2007; Youssef-Elabd, McGee et al. 2011).

Concurrent with post-prandial changes in TGs the LDL/HDL ratio reduced compared with baseline measurements. Specifically HDL was significantly reduced at time points post-prandially within all except the NOC group; potentially due to parallel elevations in chylomicrons and VLDL, as noted in other studies (Callow, Summers et al. 2002; Thomsen, Storm et al. 2003; Hanwell, Kay et al. 2009). Whilst it is established that obese T2DM patients suffer from a syndrome of high serum TG and low HDL (Laakso and Pyorala 1990), low levels of HDL are also associated with low levels of sCD14 (Eggesbo, Hjermann et al. 1994). This corresponds with the data that endotoxin has been demonstrated to bind to HDL in the presence of sCD14 and lipopolysaccharide binding protein (LBP) (Parker, Levine et al. 1995; Wurfel, Hailman et al. 1995), an enzyme involved in presentation of endotoxin to sCD14. This would support a role for HDL in immunological response to endotoxin. Therefore a reduction in HDL would reduce the removal of endotoxin further and exacerbate the inflammatory status further, compounded by higher circulating levels of endotoxin in the obese, IGT and T2DM subject groups.

Whilst our studies have highlighted the impact of metabolic disease status on circulating endotoxin, it is important to recognize the limitations of the study. In all research, it is always preferable to increase the subject numbers that comprise each cohort. In the present studies, increased numbers might have noted different post-prandial responses to the high fat meal within each cohort, if the groups were further sub-divided. However, in light of this being a



cross-sectional study, in which intra and inter comparisons can be made, the numbers do not detract from the findings. Consistent and significant trends were observed within the subjects over the 4hr post-prandial duration and differences between the cohorts duly noted.

We also recognize that the research subjects were given a very high-fat meal (75g), roughly equivalent to their total daily intake of fat, which some observers might argue is an excessive (non-physiological) amount of fat. However, despite the fat load which was based on previous studies (Ceriello, Assaloni et al. 2005), there was no significant change in LDL, HDL or total cholesterol levels (apart from Triglycerides) post-prandially in the NOC subjects, in contrast to the other groups. Furthermore administration of 75g glucose could also be considered high and would far exceed normal intake of glucose in one sitting, yet this is standard clinical practice for assessment of insulin sensitivity, whereas the fat load is only currently used as a research tool. No ill effects were noted in any of the patients during or after the study.

In summary, our current data sheds new light on our understanding of metabolic endotoxaemia in the post-prandial state in metabolic disease. Our findings suggest that circulating endotoxin levels change depending upon whether you are pre-diabetic, non-obese, obese, have IGT or have T2DM. Further that the circulating endotoxin levels noted in subjects with T2DM, at 4hr post-prandial high fat meal, far exceed our previous understanding based on other feeding studies in healthy subjects or the fasted state in T2DM subjects. Therefore, our 4hr data suggest a much higher inflammatory risk than previous studies have indicated. These findings highlight the point that requesting patients to eat smaller, more frequent meals may actually increase their inflammatory risk further, especially in subjects with T2DM - the diet of which tend to favour high fat foods (Arnold, Mann et al. 1997). Whilst vascular reactivity was affected by the high fat meal, the acute effects were less

pronounced than anticipated - which may suggest that continual feeding may yield more visible effects than one meal in isolation.

Finally, whilst the most obvious solution to metabolic endotoxinaemia appears to be to reduce saturated fat intake, the Western diet is not conducive to this mode of action and it is difficult for patients to comply with this request. Therefore we need to understand the complexity of diet, meal frequency and its acute effects on inflammatory and vascular risk and provide guidelines to particular subject groups, as leaving food intake to ‘individual preferences’ appears not to represent a beneficial solution to reduce the inflammatory state in metabolic at-risk subjects. Further studies looking at types of SFA, plant or animal based oils, food products having influence on endotoxin absorption and transport – would assist in providing individual advice for particular risk groups.

## **Chapter 4**

**Impact of endotoxaemia on sub-clinical inflammation  
and endothelial dysfunction in obese children**

## 4.1 Introduction

Environmental, physical and nutritional factors appear critical in determining lifetime disease risk profile for cardiovascular disease (CVD), which is a leading cause of mortality worldwide (Bartha, Fernandez-Deudero et al. 2012; Jankovic, Geelen et al. 2015; Reinikainen, Laatikainen et al. 2015). CVD development in later life shows an increased risk when the condition is preceded by prior chronic inflammatory conditions (John and Kitas 2012; Banerjee, Biggs et al. 2013; Eguchi and Manabe 2014; Olza, Aguilera et al. 2014; Kranendonk, van Herwaarden et al. 2015). Therefore the clinical value of determining the factors that induce an inflammatory response in early life appear important to address is compelling.

Clearly childhood obesity *per se* has a significant impact on disease risk, inflammation and CVD, however, adipose tissue (AT) may be an early contributor to metabolic dysfunction through prior systemic insults, which initiate an inflammatory response (Cook, Weitzman et al. 2003; Invitti, Guzzaloni et al. 2003; Creely, McTernan et al. 2007; Eguchi and Manabe 2014; Esser, Legrand-Poels et al. 2014; Olza, Aguilera et al. 2014; Sypniewska 2015; Zhao, Fu et al. 2015).

One cellular mechanism for an increased inflammatory response may arise through activation of the innate immune system as observed in human AT (Creely, McTernan et al. 2007; Baker, Harte et al. 2009). Previous studies have shown that increased activation of the innate immune pathway may arise through excess circulating gut derived bacteria, known as lipopolysaccharide (LPS) or endotoxin; which represents the outer cell wall membrane of gram-negative bacteria (Creely, McTernan et al. 2007; Baker, Harte et al. 2009; Piya, Harte et al. 2013). In human AT it appears endotoxin has an immediate impact on the innate immune pathway, acting via key receptors known as the toll like receptors (TLRs), which recognise

antigens such as the LPS component, to initiate an acute phase response to infection (Creely, McTernan et al. 2007; Eguchi and Manabe 2014). Stimulation of the TLRs leads to intracellular activation of NFkB, a key transcription factor in the inflammatory cascade that regulates the transcription of numerous pro-inflammatory adipocytokines (Creely, McTernan et al. 2007; Baker, Harte et al. 2009). Therefore whilst *in vitro*, endotoxin may act as a mediator of inflammation through activation of NFkB, leading to a rapid response, in an *in vivo* situation that may be further exacerbated by an increasing fat mass, such as in obesity (Dixon, Valsamakis et al. 2008; Miller, McTernan et al. 2009; Harte, da Silva et al. 2010; Harte, Varma et al. 2012; Li, Ma et al. 2015).

Clinical studies have also implicated gut derived endotoxin as a direct ‘primary mediator’ to activate the inflammatory state, contributing to metabolic disease, with current cross sectional data showing elevated systemic endotoxin levels in conditions of obesity, coronary artery disease, type 2 diabetes mellitus and fatty liver disease (Creely, McTernan et al. 2007; Piya, Harte et al. 2013; Eguchi and Manabe 2014), which is reduced with weight change (Dixon, Valsamakis et al. 2008; Harte, da Silva et al. 2010).

Studies in adults has also shown circulating endotoxin to be positively associated with waist, waist-hip-ratio, insulin levels, inflammatory cytokines as well as lipids, including total cholesterol, triglycerides, LDL-cholesterol and negatively associated with HDL-cholesterol (Dixon, Valsamakis et al. 2008; Miller, McTernan et al. 2009; Harte, da Silva et al. 2010; Harte, Varma et al. 2012; Eguchi and Manabe 2014; Li, Ma et al. 2015).

Recent studies in obese children and adolescents has demonstrated that systemic inflammatory cytokines such as plasminogen activator inhibitor-1 (PAI-1) and C-reactive protein (CRP) are elevated, along with vascular injury and atherogenesis markers, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule type-1

(ICAM-1) (Koning 2015; Lovren, Teoh et al. 2015). Whether such pathogenic biomarkers directly correlate with systemic endotoxin concentrations in obese children and adolescents is undetermined. Therefore the aim of this study was to examine bacterial endotoxin as a potential biomarker of sub-clinical inflammation and early CVD risk in childhood obesity.

## 4.2 Methods and Materials

### 4.2.1 Study subjects

A total of 60 (unless stated otherwise in the figure legend) obese children and adolescents with varying degrees of obesity (Body Mass Index) (BMI: mean $\pm$ (SD) 35.1 $\pm$ 5.2kg/m<sup>2</sup>; age: 13.9 $\pm$ 2.3 years) were recruited among those referred for weight loss intervention to the obesity centre of the Istituto Auxologico Italiano. All subjects were above the age and sex adjusted 97<sup>th</sup> BMI percentile, which defines obesity according to the Italian BMI charts (Cacciari, Milani et al. 2002) and had an age range of 8-18 yrs. The Ethics Committee of the Italian Institute approved this study and informed consent was obtained from all subjects and their parents. All subjects underwent an oral glucose tolerance test (1.75 g/Kg, up to a maximum of 75 g glucose in 250 ml of water) following an overnight fast. Plasma samples were drawn at baseline, after 30 min and 120 min, for determination of plasma glucose and insulin concentration. Categorisation of glucose tolerance status was made using the World Health Organisation criteria (Li, Ma et al. 2015). The impaired fasting glucose was defined by fasting glucose levels  $\geq$ 5.6 mmol/l (M.Nathan 2007; Nathan, Davidson et al. 2007). Blood samples were drawn for measurement of endotoxin, adiponectin and, markers of inflammation and CVD. Blood pressure measurements were taken as previously described (Invitti, Guzzaloni et al. 2003). Insulin resistance was measured by HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) (fasting insulin x fasting glucose/22.5) (Matthews, Hosker et al. 1985).

#### 4.2.2 Biochemical Measurements

Serum endotoxin was assayed using a Chromogenic Limulus Amebocyte Lysate (LAL) test, which is a quantitative test for gram-negative bacterial endotoxin (Cambrex, New Jersey, USA) endotoxin-free vials were utilised throughout. Gram-negative bacterial endotoxin catalyzes the activation of a pro-enzyme in the Limulus Amebocyte Lysate (LAL). The initial rate of activation is directly determined by the concentration of endotoxin. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405-410 nm following termination of the reaction. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/mL range. Intra-assay CV  $3.9 \pm 0.46$ , inter-assay CV  $9.6 \pm 0.75$ . For the purposes of these studies all samples were run in duplicate within the same plate, therefore no inter-assay variability was observed in this study. To assess recovery of endotoxin within the assay, previous studies have utilised known concentrations of recombinant endotoxin (0.25 and 1.00 EU/mL) were added to diluted, pooled plasma to determine whether the expected concentration correlated closely with the actual observed value and whether there were any variations due to reaction with plasma contents [16]. Lyophilized endotoxin (*E. coli* origin) was used to generate a standard curve with the Chromogenic LAL test kit from Cambrex and produced a corresponding curve in accordance with the manufacturer's instructions. In plasma, the recovery of spiked endotoxin was  $82.0 \pm 3.3\%$  efficient, similar recovery data were noted for serum. Plate to plate variability within the same experiment was  $7.4 \pm 0.9\%$ , these findings were similar to those observed from assessment by Cambrex (Creely, McTernan et al. 2007).



A multiplexed CVD Panel 1 immunoassay (Linco Research, Missouri, USA) was utilised to examine the circulating concentrations of the following inflammatory and CVD risk biomarkers: (Tumour Necrosis factor- $\alpha$ ) TNF- $\alpha$ , PAI-1 (tPAI-1, total) CRP, soluble intercellular adhesion molecule type-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), MMP-9, MPO (Myeloperoxidase), VEGF (Vascular Endothelial Growth Factor) and soluble endothelial selectin (sE-Selectin). The CVD Panel 1 immunoassay had a sensitivity of 16-50,000 pg/ml for (Matrix MetalloProteinase-9) MMP-9, MPO and PAI-1 (Plasminogen Activator Inhibitor – 1) and further, a sensitivity of 80-250,000 pg/ml for sICAM-1 (Soluble Intercellular Adhesion Molecule Type-1), sVCAM-1 and sE-Selectin; with an intra- and interassay CV (co-efficient of variation) of 4.5-12.3% and 8.5-16.3%, respectively. Plasma glucose was measured using an automated glucose analyser (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured by a chemiluminescent assay (DPC, Los Angeles, USA) with a sensitivity of 14.3 pmol/l and intra- and interassay CV of 3.7% and 6.7%, respectively.

#### 4.2.3 Statistical Analysis

All analyses were performed using statistical software (SPSS, version 14; Woking, UK). Variables that were not normally distributed were log transformed. Differences between groups were calculated using a unpaired t-test for independent samples. A Pearsons' correlation analysis was used to analyse bivariate relationships between endotoxin and the various markers of inflammation and vascular injury. Data were expressed as mean  $\pm$  SD. A p-value  $<0.05$  was considered statistically significant.

## **4.3 Results**

### **4.3.1 Effect of gender on Biomarkers of Inflammation**

From this cohort, biochemical analysis was performed on 24 male subjects and 36 female subjects with matching BMI and age. Clinical and biochemical characteristics of male and female obese subjects are provided in Table 4.3.1.

Clinical and Biochemical Characteristics	Male Subjects ( $\pm$ SD)	Female Subjects ( $\pm$ SD)	p value
Age (yrs)	14 $\pm$ 3	14 $\pm$ 2	-
BMI (kg/m <sup>2</sup> )	34.0 $\pm$ 5.1	35.6 $\pm$ 5.3	-
Fasting Glucose (mmol/l)	4.4 $\pm$ 0.3	4.5 $\pm$ 0.5	N/S
2 h glucose, mmol/l	6.0 $\pm$ 1.0	5.7 $\pm$ 1.0	N/S
HOMA-IR	2.6 $\pm$ 1.2	3.3 $\pm$ 2.1	N/S
Endotoxin (EU/ml)	10.1 $\pm$ 5.4	5.3 $\pm$ 3.7	p<0.01**
CRP ( $\mu$ g/ml)	7.5 $\pm$ 8.3	9.0 $\pm$ 7.5	N/S
TNF- $\alpha$ (pg/ml)	5.0 $\pm$ 2.5	5.9 $\pm$ 5.8	N/S
PAI-1 (ng/ml)	27.0 $\pm$ 10.7	26.0 $\pm$ 15.7	N/S
sICAM-1 (ng/ml)	110.4 $\pm$ 37.4	103.1 $\pm$ 47.9	N/S
Systolic BP (mmHg)	121.0 $\pm$ 10.8	119.1 $\pm$ 8.5	N/S
Diastolic BP (mmHg)	74.0 $\pm$ 11.2	72.7 $\pm$ 8.5	N/S

Table 4.3.1 Clinical and biochemical characteristics for obese, BMI and age-matched male (n=24) and female (n=36) subjects. Data are expressed as mean $\pm$ SD. Significant differences in data between male and female subjects are highlighted (P-Values: \*, p<0.05, \*\*, p<0.001).

No significant gender differences were observed in HOMA-IR, blood pressure or several markers of inflammation and CV (cardiovascular) injury.

### 4.3.2 Correlation of Endotoxin with Biomarkers of Inflammation and CVD in Childhood & Adolescent Obesity

In this study circulating endotoxin concentrations significantly and positively correlated with TNF- $\alpha$  ( $p<0.05$ ,  $r^2=0.077$ ) and MCP-1 (Monocyte Chemoattractant Protein-1) ( $p<0.01$ ,  $r^2=0.178$ ) (Figure 1A and 1B). However, no significant correlation was noted between circulating endotoxin levels and CRP ( $p=N.S$ ,  $r^2=-0.069$ ).

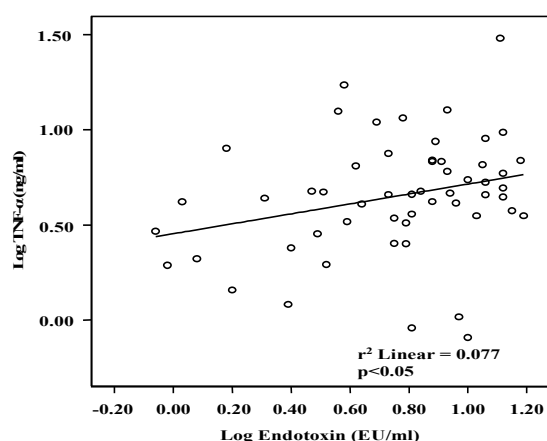


Figure 1A

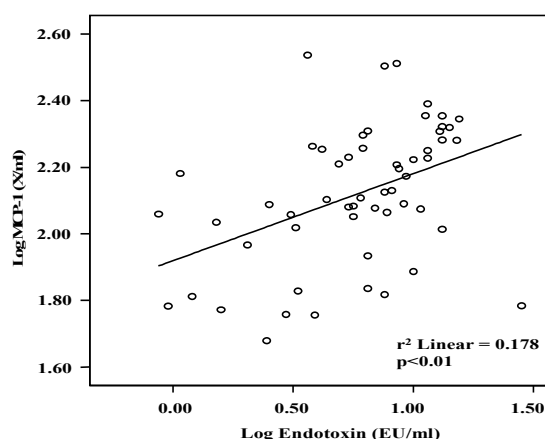


Figure 1B

Figure 1A and 1B. Correlation between log endotoxin (EU/ml) levels and the inflammatory markers: (A) log TNF- $\alpha$  pg/ml ( $p<0.05$ ) (B) log MCP-1 ng/ml ( $p<0.01$ ), in BMI and age-matched children and adolescents.

With regards to CVD risk markers, circulating endotoxin concentrations further correlated with several parameters of atherogenesis and vascular injury; these included PAI-1 ( $p<0.01$ ,  $r^2=0.215$ ), sICAM-1 ( $p<0.01$ ,  $r^2=0.159$ ), MMP-9 ( $p<0.01$ ,  $r^2=0.159$ ), MPO ( $p<0.05$ ,  $r^2=0.07$ ) and VEGF ( $p<0.01$ ,  $r^2=0.161$ ) (Figure 2A-F).

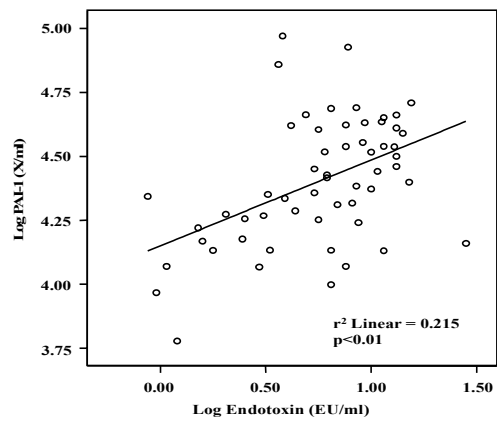


Figure 2A

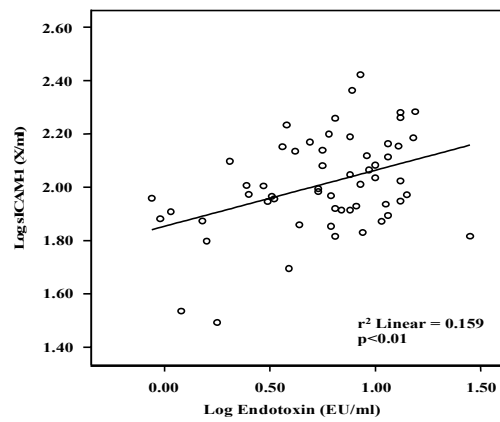


Figure 2B

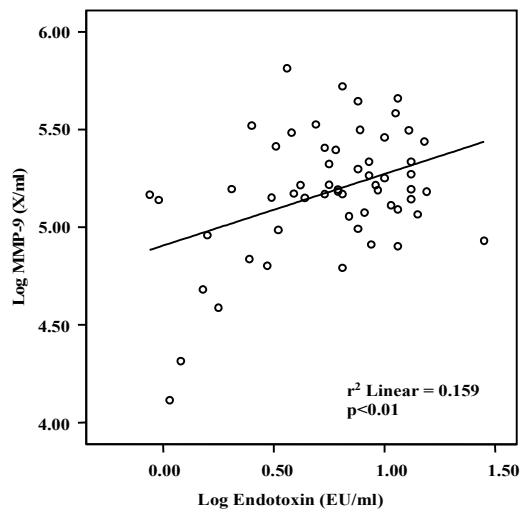


Figure 2C

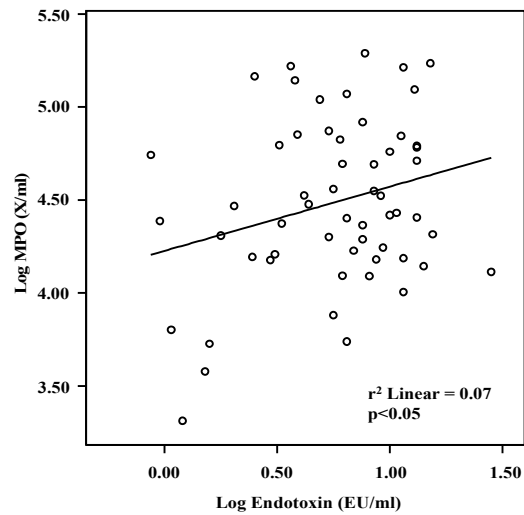


Figure 2D

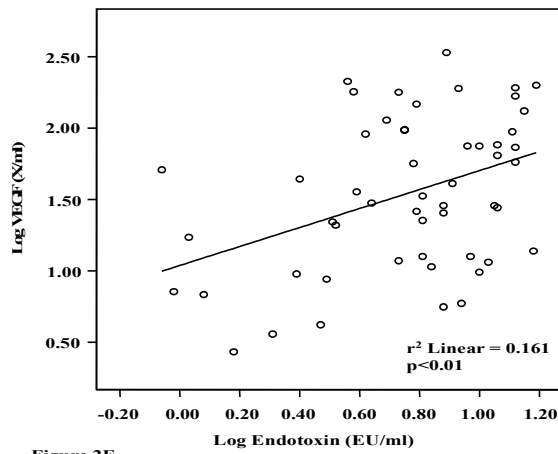


Figure 2E

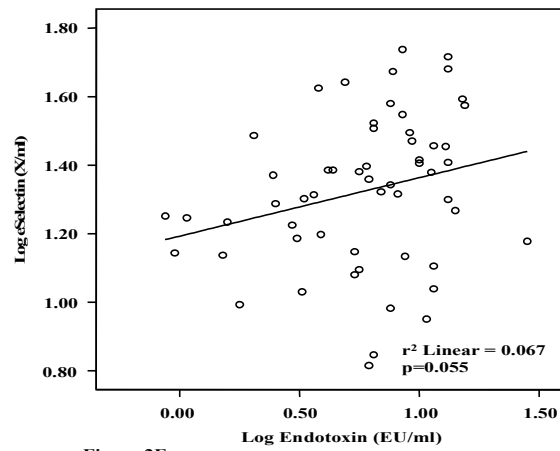


Figure 2F

Figure 2A-F. Correlation between log endotoxin (EU/ml) levels and the following markers of CVD: (A) log PAI-1 (ng/ml) ( $p < 0.05$ ), (B) log sICAM-1 (ng/ml) ( $p < 0.05$ ), (C) log MMP-9 (pg/ml) ( $p < 0.05$ ), (D) log MPO (pg/ml) ( $p < 0.05$ ), (E) log VEGF (pg/ml) ( $p < 0.05$ ) and (F) log eSelectin (X/ml) ( $p < 0.05$ ) in BMI and age-matched children and adolescents.

No significant correlation was observed between endotoxin and eS-Selectin levels ( $p = 0.055$ ,  $r^2 = 0.067$ ) or sVCAM-1 levels ( $p = \text{N.S.}$ ,  $r^2 = 0.054$ ).

Additional analysis of serum concentration data revealed that circulating endotoxin levels positively correlated with systolic blood pressure ( $p < 0.05$ ;  $r^2 = 0.155$ ;  $n = 37$ ) (Figure 3A) and diastolic blood pressure ( $p < 0.05$ ;  $r^2 = 0.083$ ;  $n = 51$ ) (Figure 3B).

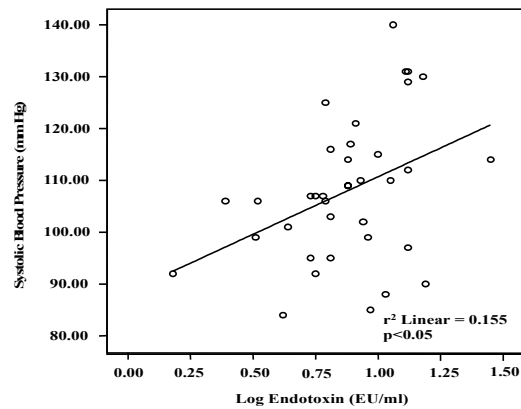


Figure 3A

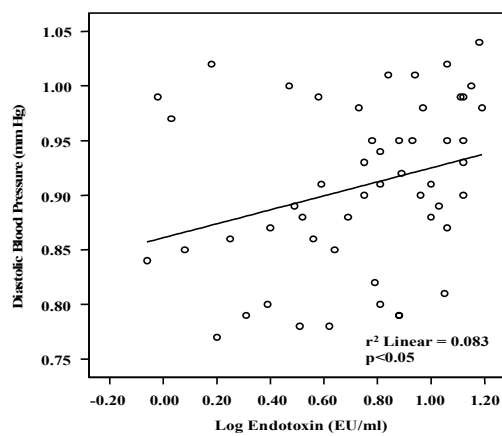


Figure 3B

Figure 3A and 3B. Correlation between log endotoxin (EU/ml) levels and (A) systolic blood pressure (mm Hg) (n=37) and (B) diastolic blood pressure (mm Hg) (n=51) in BMI and age-matched children and adolescents.

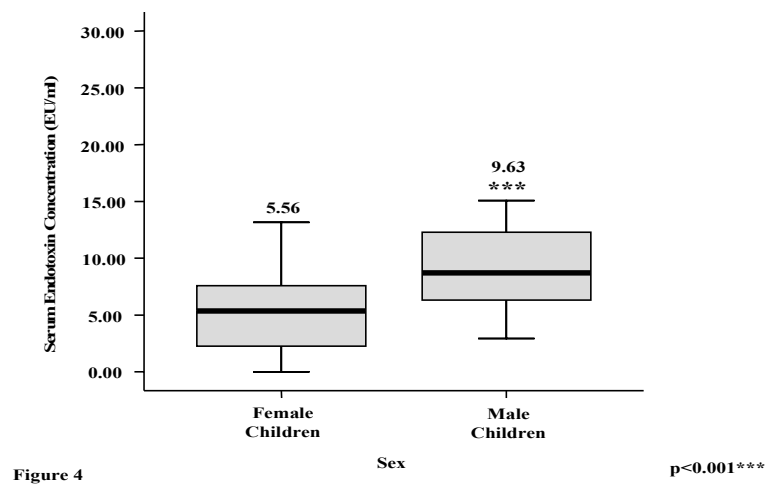


Figure 4. Comparison of the mean relative endotoxin serum concentrations (EU/ml)  $\pm$  SEM in male and female BMI and age-matched children and adolescents.

Endotoxin was higher in males than females as a direct comparison (Figure 4).



## 4.4 Discussion

These current studies highlight that in childhood obesity circulating endotoxin is significantly correlated with pro-inflammatory markers, TNF- $\alpha$ , MCP-1, as well as biomarkers of atherogenesis and vascular injury including PAI-1, sICAM-1, MMP-9 and MPO, VEGF. Furthermore, data analysis also determined that circulating endotoxin levels are positively correlated with both systolic and diastolic blood pressure in children with obesity. Taken together these findings also indicate that the presence of metabolic endotoxinaemia, which appears to occur in childhood, correlates with pathogenic pro-inflammatory factors in a similar manner to that noted in adults with metabolic disease (Creely, McTernan et al. 2007; Dixon, Valsamakis et al. 2008; Baker, Harte et al. 2009; Harte, da Silva et al. 2010). These studies also appear to suggest that the raised endotoxin levels in childhood are coupled to a noted high disease risk profile and blood pressure, which together ultimately could lead to an earlier life progression of CVD. Endotoxin may also account for, in part, the continual pro-inflammatory state experienced in obese children (Gilardini, McTernan et al. 2006; Al-Attas, Al-Daghri et al. 2010; Kheirandish-Gozal, Peris et al. 2014; Chang, Jian et al. 2015).

These studies also observed for the first time a noted significant rise in circulating endotoxin in boys compared with girls. Whilst this was not the aim of the study these findings appear consistent with gender-specific effects noted in adults; maintained across several ethnicities (Miller, McTernan et al. 2009). Prior studies in adults suggest a higher endotoxin-induced pro-inflammatory cytokine release in men than women, although this study in children did not identify this gender specific aspect (Willerson and Ridker 2004; Dandona, Aljada et al. 2005). Such a disparity in endotoxin-induced pro-inflammatory cytokine release between the children and adults with obesity may arise, in part, due to the difference in exposure time to endotoxin; which in obese adults may give rise to long-term damaging inflammatory change

leading to T2DM (Matthews, Hosker et al. 1985; Gilardini, McTernan et al. 2006; Nathan, Davidson et al. 2007; Temple, Pham et al. 2008; Smit, Heederik et al. 2009; Al-Attas, Al-Daghri et al. 2010; Bhatia, Sekhon et al. 2014; Kheirandish-Gozal, Peris et al. 2014; Chang, Jian et al. 2015).

These studies also suggest that changes in inflammation, vascular dysfunction, and blood pressure in childhood obesity may arise beyond the known impact of the cardiometabolic lipid profile (Dandona, Aljada et al. 2005; Gilardini, McTernan et al. 2006; Nadeau, Maahs et al. 2011; Chang, Jian et al. 2015). A key mediator to increase disease risk arise from circulating commensal bacterial endotoxin, derived from the gastrointestinal-tract eliciting a pro-inflammatory response in prior childhood and adolescent obesity studies (Pussinen, Havulinna et al. 2011; Assimakopoulos, Tsamandas et al. 2013). Additionally, adult studies highlight that diets high in fat and or processed meat appear to raise endotoxin levels further, whilst dairy products and other food combinations may reduce endotoxin levels and inflammation (Deopurkar, Ghanim et al. 2010; Harte, Varma et al. 2012; Schwander, Kopf-Bolanz et al. 2014; Schmid, Petry et al. 2015). As such further future insight into examining the impact of diet on endotoxin levels in children with obesity may highlight important interventions to reduce the long-term health risk (Creely, McTernan et al. 2007; Lira, Rosa et al. 2010; Assimakopoulos, Tsamandas et al. 2013; Mischke and Plosch 2013; Kellow, Coughlan et al. 2014).

Several risk factors in childhood have been proposed to predict the later development of CVD including obesity, hypertension and endothelial dysfunction promoting atherogenesis and thrombosis (Skurk and Hauner 2004; Cote, Harris et al. 2013; de Koning, Denhoff et al. 2015; Ramji and Davies 2015). In our cohort of obese children and adolescents, several biomarkers of vascular injury and endothelial dysfunction, including PAI-1, sICAM-1, MMP-9, MPO

and VEGF, were significantly and positively correlated with circulating endotoxin concentrations. The observed pro-inflammatory biomarker risk profile in the obese children appears to be similar to both what has been identified in adults with CVD as well as studies comparing obese and lean children (de Koning, Denhoff et al. 2015).

Hypertension has been identified as a key risk factor for atherogenesis and vascular injury, and this study noted a positive association between endotoxin with systolic and diastolic blood pressure. As such a diet that raises endotoxin levels would appear to also increase blood pressure. Whilst this is a cross-sectional study, which therefore cannot determine a causal relationship, in previous studies have demonstrated that mice fed a continuous endotoxin bolus, exhibited a subsequent increase in vascular dysfunction (Hao, Zhang et al. 2010; Sordi, Chiazza et al. 2015). This is consistent with the concept that an endotoxin-induced inflammatory response in childhood obesity, may consequently contribute to an accelerated risk of CVD in later life. Furthermore the noted correlations between circulating endotoxin and biomarkers of endothelial dysfunction, in this childhood obesity study, could also promote a cluster of these pro-atherogenic factors contributing to accelerated atherosclerosis, arterial stiffness and CVD in later life (Thompson, Obarzanek et al. 2007; Nadeau, Maahs et al. 2011; Prasad, Kabir et al. 2011; Armstrong, Cote et al. 2014; Cote, Phillips et al. 2015).

This study had some limitations, namely: the cross-sectional design which did not allow causal determinations and a limited sample size indicating modest correlations. Further, the lack of gender-effects on the correlations between endotoxin and the pro-inflammatory factors may also be considered a reflection of the limited cohort size. Thus, future studies exploring further aspects of these observations in larger cohorts are warranted.

In summary, this study highlights the relationship between endotoxin and several inflammatory and CVD risk biomarkers, particularly as an early player in obesity-related inflammatory disorders during childhood and adolescence even though we did not correlate endotoxin with age.

Furthermore, even at an early age in the obese state, female subjects exhibited lower endotoxin levels than their male counterparts, suggesting a more favourable metabolic profile. This may manifest in later life as delayed CVD mortality and morbidity for females in contrast to males. The reason for such a difference is not clearly known even though dietary and hormonal differences could be speculated. Finally, among the inflammatory markers evaluated in this study, endotoxin may serve as a potential mediator of sub-clinical inflammation in childhood and adolescent obesity as noted in adult studies.

## **Chapter 5**

**Assessment of ED using Non invasive vascular function in**

**Healthy subjects**

## 5.1 Introduction

This chapter is intended to validate 2 newer technologies of FMD brachial artery measurement and RVA retinal artery measurements in healthy subjects using Glucose ingestion as physiologic stress. The next chapter uses the same healthy cohort when comparing T2DM subjects for some parameters.

### 5.1.1 Endothelium

The endothelium is a specialized layer of thin mononuclear cells that covers the inner surfaces of all blood vessels. It forms a barrier across the circulating blood from the vascular smooth muscle and tissues. In healthy subjects, unperturbed vascular endothelium functions as an athero-protective organ through vasoactive mediators such as nitric oxide (NO), prostacyclin, and endothelium derived relaxation factor (EDRF). It senses mechanical stimuli, such as pressure and sheer stress and responds to both exocrine and paracrine hormonal stimuli. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect homeostasis (Drexler 1998; Esper, Nordaby et al. 2006; Deanfield, Halcox et al. 2007; Shimokawa and Godo 2015). However, in subjects with cardiovascular disease (CVD) or indeed any vascular disease, homeostasis of the functional endothelium is compromised resulting in endothelial dysfunction.

### 5.1.2 Endothelial dysfunction

Normal endothelial function is crucial in maintaining cardiovascular health. Impairment causes physiological derangement of the vascular tone, inflammation and thrombosis. Thus,

dysfunctional endothelium loses its protective effect on the vasculature. It is now accepted as one of the first quantifiable steps in the process of progressive atherosclerosis (Drexler and Hornig 1999; Endemann and Schiffrin 2004; Esper, Nordaby et al. 2006; Deanfield, Halcox et al. 2007) and it can predate clinically obvious vascular pathology by many years (Benzuly, Padgett et al. 1994; Vlahos, Naka et al. 2014). Assessment of endothelial dysfunction has also been shown to be of prognostic significance in predicting vascular events (Brevetti, Silvestro et al. 2003), including heart attacks and stroke (Celermajer, Sorensen et al. 1994; Neunteufl, Heher et al. 2000; Schächinger, Britten et al. 2000; Chan, Mancini et al. 2003; Quyyumi 2003; Fichtlscherer, Breuer et al. 2004; Frick, Suessenbacher et al. 2005; Yoshida, Kawano et al. 2006; Yeboah, Crouse et al. 2007). A key feature of this phenomenon is the inability of vasculature to react appropriately, thereby inducing a pro-inflammatory, pro-thrombotic state and reduced vasodilatation in response to appropriate stimulus (Sukhovshin, Yepuri et al. 2015). Dysfunctional endothelial cells are unable to produce NO to the same extent as healthy endothelial cells and therefore their ability to react and dilate to stress is reduced (Vallance and Chan 2001; Anderson 2003). This imbalance of homeostasis in endothelial function creates a detectable difference in subjects with endothelial dysfunction verses individuals with normal healthy endothelium.

The study therefore assessed the vascular endothelial function of healthy non diabetic (ND) subjects using post-prandial glucose test as stress. The focus of this chapter was to assess the macrovascular endothelial function initially and its response to glucose challenge/stress in ND subjects. Secondly, this study examined similar effects of glucose challenge on microvascular function in the same cohort of subjects. Macrovascular dysfunction, as elicited by (flow mediated dilatation) FMD, has already been shown to be an important predictive factor not only for atherosclerosis (Ross 1993), but also for future CVD (Celermajer,

Sorensen et al. 1992; Gokce, Keaney et al. 2002; Vita and Keaney 2002; Gokce, Keaney et al. 2003; Yeboah, Crouse et al. 2007).

This study would attempt to re-affirm previous data using a more advanced, validated and accurate software to measure endothelial function. The study would then investigate the effects of physiologic stress on the endothelial function at both macrovascular and microvascular levels in ND subjects. Microvascular function would be evaluated utilizing a newer system of Dynamic vessel analysis (DVA) of retinal vessels. Finally, an attempt would be made to compare and contrast both systems in the same groups.

## **5.2 Materials and methods**

### **5.2.1 Demographics**

The study was performed at UHCW NHS Trust on a group of ND subjects following initial recruitment from their primary care practices. A total of 10 ND subjects were recruited into the study. All ND subjects were of South Asian Origin except one who was Afro- Caribbean descent. The diagnosis of ND status was made according to the 2006 WHO criteria with fasting glucose level of  $\leq 7.0$  mmol/l. The clinical characteristics of the group are reported below in (table 5.2.1).

The subjects were drug naïve and therefore not under the influence of any exogenous substances which could potentially influence endothelial function.



ND / Healthy subjects	Mean	Std. Error (SEM)
Age	39.8	3.6
Sex male/ female n	7/3	-
BMI, Kg/m <sup>2</sup>	25.4	1.0
Fasting glucose, mmol/l	5.2	0.2
Glycated Heamoglobin %	5.8	0.1
Diastolic blood pressure	71.6	2.4
Total cholesterol, mmol/litre	5.1	0.3
HDL, mmol/litre	1.4	0.1
LDL, mmol/litre	3.1	0.2
TG, mmol/litre	1.3	0.2
Mean FMD %	3.7	1.6
Mean RVR %	3.2	0.5

Table 5.2.1 ND subjects' demographic profile and baseline characteristics

Data are expressed as means $\pm$  SEM (standard error of mean). As noted in table 5.2.1, the mean BMI for the group is favourable at 25. Similarly, the mean resting diastolic blood pressures and fasting glucose were all within acceptable normal limits.

### 5.2.2 Patient Recruitment and selection

Only non-smoking subjects with a normal resting ECG and blood pressure were selected for the study. The full list of inclusion and exclusion criteria is covered in detail in chapter 2. Subjects on aspirin, statins, or supplemental vitamins were excluded - as they have been shown to influence endothelial function (Drexler and Hornig 1999; Ceriello, Taboga et al. 2002). All subjects were recommended to consume their habitual diet during the entire period of the study. A validated dietary questionnaire was utilised to obtain dietary habits. Subjects were recruited once they fulfilled all the inclusion and exclusion criteria for the study. None of the subjects had any history of retinopathy or renal dysfunction.

### 5.2.3 Clinic Visit

The initial clinic visit was undertaken in the mornings to facilitate fasting blood tests. Full Ethical approval was obtained for the conduct of the study from the local Research Ethics committee. Specific details regarding organization and conductance of the clinic visits were covered in detail in chapter 2. Once at the clinic, all efforts were made to explain the study protocol in the subjects' own language. After informed consent and enrolling into the study, fasting blood and urine samples were obtained. Screening blood tests were also performed for both baseline measurements to qualify for the study, as well as to assess their glucose control. Routine blood tests included renal functions, glucose, HbA1C and full cholesterol profile, which were subsequently sent to the UHCW biochemistry laboratory for analysis. All subjects then had their height, weight, abdominal circumference and BMI measurements taken using the same standard equipment. Blood samples were taken using standard technique either from the right or left ante- cubital vein in a sitting position. Blood pressure was measured using an automated sphygmomanometer on the left arm and rechecked after 2 min.

Finally a 12 Lead ECG was performed using the same machine for the duration of the study. Female chaperones were utilized during the clinics as deemed appropriate.

#### 5.2.4 Study protocol

Vascular function tests (as detailed below) were performed and blood samples were drawn at 0 (fasting state), 1 (1 hr post stress), 2 (2 hr post stress), 3 and 4 hr post glucose stress. The glucose stress was a standard oral glucose tolerance test which involved taking seventy five (75) grams of glucose as a drink. The quantity of glucose utilized is standard and according to existing published literature (Ceriello, Assaloni et al. 2005). Serum glucose, full lipid profile, insulin and endothelial markers were also assayed along with the vascular function tests at each time point, as described above. Vascular function tests involved testing endothelial function at the level of the arm and retina. These tests utilized ultrasound techniques to measure flow-mediated vasodilatation (FMD) of the brachial artery and, similarly, retinal vessel reactivity (RVR) using a Retinal vessel analyzer (RVA).

#### 5.2.5 Serum and plasma isolation

Using the standard procedure serum and plasma was isolated during the clinic visit. Once blood was drawn and into separate tubes, the tubes collecting serum and plasma were independently stored on ice and transported to the research laboratory, for storage, based at the Clinical Sciences Research Institute Laboratories (CSRI) at UHCW NHS Trust, Coventry. The detailed procedure is described in chapter 2.

### **5.3 Vascular Function Tests**

The following details describe the status of vascular inner lining membrane, the endothelium.

#### **5.3.1 Endothelial Function at macrovascular level**

Endothelial function at macrovascular level was evaluated by measuring flow-mediated vasodilatation (FMD) of the brachial artery. FMD is a non-invasive method of assessing the endothelial function. The specific details of the test are explained in chapter 2. The examination was carried out in a temperature and light controlled room on subjects, who were lying comfortably flat on a couch (Appendix I, Figure 1 f). The temperature was constantly maintained at 21° C using an efficient central air conditioner. A BP cuff was placed on the left arm, which was connected to a quality checked and NHS (National Health Service) approved automated BP machine, that was used to check systolic and diastolic BP at various times during the study protocol. The same machine was used in all study subjects during the entire duration to reduce any possible variations between machines. ECG tracing was obtained by 3 leads connected to the VIVID 7 ECHO machine (GE Vingmed System V) (Appendix I, picture 1 g). The details of endothelial function testing are covered in chapter 2.

#### **5.3.2 Flow mediated dilatation (FMD) Measurements**

In this study, brachial arteries were imaged with a standard ultrasound system (VIVID 7 ECHO machine (GE Vingmed System V) connected with a 12 MHz flat linear transducer probe. The ultrasound system was connected to a personal computer equipped with a frame grabber and artificial neural network wall detection software (vessel image analysis) (VIA)

(Sidhu, Newey et al. 2002), as described in chapter 2. The VIA software automatically detects and tracks the anterior and posterior walls within a user defined region of interest (Sidhu, Newey et al. 2002). The details of the procedure and the protocol used are described in chapter 2.

The endothelium independent dilatation of the brachial artery was calculated similarly to the percentage increase in mean diameter 5 min after glyceryl trinitrate administration. The FMD was calculated and displayed within seconds of completing the study, using VIA software, as previously described. This new technique has previously been shown to minimise the variability of measurement (Sidhu, Newey et al. 2002). Using a reference two dimensional image of the artery for comparison in repeat studies also ensured that the same section of artery was scanned every time and on subsequent days. Similar measurements to detect endothelial function were undertaken in the retinal vasculature in order to study microvascular function. For this part of the study, the following procedure was used.

### 5.3.3 Retinal Vessels Reactivity Measurements

Observations and measurements in retinal vessels detect microvascular changes in a direct and non-invasive method. This method is used by clinicians using an ophthalmoscope to identify changes in the vascular bed relating to diabetes, hypertension and other cardiovascular risk parameters. However, early functional vascular changes due to diabetes and hypertension could occur without any identifiable or obvious lesions on traditional ophthalmoscope examination (HICKAM and SIEKER 1960). Similarly, early functional changes in the retinal vascular bed may not be identifiable on current retinal screening programmes based on identifying structural abnormalities. Therefore, in order to identify these early functional vascular changes of vasosclerosis (when there is little or no change on

ophthalmoscope examination (HICKAM and SIEKER 1960) or retinal screening fundal photographs), the RVA, with the ability to objectively measure vascular function, was utilized to measure microvascular function in this study.

RVR was measured using the RVA (Imedos, GmbH). The fundamental components of this system consisted of a fundus camera (field of 50°; model FF 450; Zeiss, Jena, Germany), a video camera, a real-time monitor, and a computer with image-analysis software for the accurate determination of retinal arterial and venous diameter. The details of the system were explained in chapter 2.

#### 5.3.4 Mechanism of wall motion detection-Retinal Vessel Analyser

The RVA system, as a method suitable for quantitative examination of retinal vascular function, has been previously validated in a clinical methodological study by Nagel (Nagel and Vilser 2004). The RVA methodology and system information is described in detail in chapter 2.

#### 5.3.5 Retinal Vessel Analyser (RVA) Protocol

In brief, all subjects had 1 % Tropicamide applied, usually to the right eye, 15-30 min before the actual scanning for pupillary dilatation. The subjects were seated before the fundal camera and the stool height was adjusted so that the subject was comfortable for the duration of the study. The image of the retina was adjusted on the screen of the real-time monitor. A suitable area in the retina was picked for scanning after adjusting the camera to the dilated pupil. Once a clear fundus image with good contrast was obtained, the scanning commenced according to

a fixed protocol. The fundus of the eye was examined under green light. (The details of this protocol are discussed in detail in chapter 2). A graph, similar to the one in the example noted in (Appendix II, Figure 2 c and Figure 2 d) , was obtained at the end of scanning. This figure reveals the vascular reactivity result on-line, instantly. The results are shown below.

## 5.4 Results

### 5.4.1 Physiological stress response

Baseline plasma glucose levels increased significantly over time following ingestion of 75 grams glucose, as part of OGTT. The increased glucose levels reverted back to baseline levels by 3 to 4 hrs. The table below indicates that mean baseline values increased only modestly following the OGTT, in keeping with efficient glucose metabolism in healthy individuals.

Glucose stress ND	Mean serum glucose	Std. Deviation
Fasting	5.2	0.6
1 hr. post	6.8	2.3
2 hr. post	6.0 *	2.5
3 hr. post	4.2 *	0.6
4 hr. post	4.0 *	0.4

Table 5.4.1.1 Serum glucose levels following OGTT on ND subjects \*  $P < 0.05$

The results shown in table 5.4.1.1 demonstrate significant changes to glucose levels at baseline compared with the post-glucose stress time points (1-4hr) following OGTT in ND subjects. During this time, the plasma triglyceride (TG) levels revealed a stable trend (table 5.4.1.2).



Glucose stress ND	Mean serum TG	Std. Deviation
Fasting	1.3	0.6
1 hr. post	1.1	0.6
2 hr. post	1.4	1.6
3 hr. post	1.1	0.8
4 hr. post	1.2	0.9

Table 5.4.1.2 Serum TG levels following OGTT in ND subjects

These findings confirm that, following OGTT, there is no significant change to TG levels compared with baseline in ND / healthy subjects.

The levels remained consistent throughout the 5 hours of testing, including the fasting phase and during an extended OGTT phase. This finding demonstrates that TG levels are not influenced by glucose ingestion, which could also be interpreted as a validation of the glucose challenge test, as expected.

#### 5.4.2 Macrovascular response to OGTT

Flow mediated dilatation response (macrovascular response) of the brachial artery to physiologic stress is detailed below (table 5.4.2).

Glucose stress ND	Mean FMD %	Std. Error
Fasting	3.7	1.1
1 hr. post	1.6	0.8
2 hr. post	0.8 *	0.9
3 hr. post	2.5	0.5
4 hr. post	2.1	1.0
Nitroglycerine spray	14.6 **	2.5

Table 5.4.2 FMD of brachial artery response to OGTT in ND subjects

\*  $P < 0.05$  \*\*  $P < 0.01$

As noted in Table 5.4.2, mean baseline flow mediated dilatation (FMD) of the brachial artery in ND subjects was 9.8%. This value is significantly higher than the mean baseline levels noted in T2DM subjects (as revealed in detailed in chapter 6).

At 1 hour post OGTT, the mean FMD significantly and profoundly reduced to 1.6 %. At hours 2 and 3 after the OGTT, the FMD started to recover very slowly, thus revealing the acute effects of post-prandial glucose stress on the healthy endothelium.

In contrast to the above endothelial dependant depressive effect noted with acute hyperglycaemia, FMD response levels of 14.6 % were seen upon administration of Sublingual nitroglycerin (GTN, 250 µgrams). This level of dilatation (response) noted after GTN spray is statistically significant and is indicative of non-endothelial dependant dilatation

secondary to smooth muscle relaxation. This effect appears to be independent of endothelial influence on the vascular reactivity.

#### 5.4.3 Microvascular response to OGTT

Retinal vessel diameter measurements were represented as percentages similar to FMD brachial artery measurements. Microvascular (retinal vessel reactivity-RVR) response to OGTT is shown below (5.4.3).

Glucose stress ND	Mean RVR %	Std. Error
Fasting	3.2	0.5
1 hr. post	3.3	0.5
2 hr. post	2.7	0.2
3 hr. post	2.6	0.3
4 hr. post	3.3	0.5

Table 5.4.3 RVA of retinal artery in response to OGTT in ND subjects

As noted in Table 5.4.3, the mean baseline retinal vessel reactivity (RVR) in ND subjects during the fasting state was 3.2 %. It is significantly high compared with T2DM subjects (as shown in chapter 6). At 1 hr post glucose ingestion, the mean RVR increased to 3.3 %. This trend of increasing reactivity measurements to stress was quite different from the reducing

reactivity measurements of the macrovasculature, as represented by brachial artery reactivity. At 2 and 3 hr post-glucose stress, the reactivity measurements started to reduce to even below the baseline value. This dynamic trend of an initial increase before a gradual depression, in terms of arterial reactivity in the microvasculature, seemed to follow a similar pattern to that of blood glucose levels - which reached a peak at hour 1 before regressing. It is therefore, likely that this initial high reactivity could be related to high glucose levels acting as a stimulus.

#### 5.4.4 Summary

In summary, following the OGTT in healthy ND subjects, serum glucose levels increased to a peak at 1 hr before gradually reducing back to pre-prandial levels. The levels reached statistical significance at hr 2, 3 and 4. At the same time, serum TG levels did not change significantly, which supports the validity of the OGTT for this group of subjects. In a recent study, it is quite clear that FMD does not significantly change to usual physiologic changes during the morning in healthy people (Thosar, Wiggins et al. 2015). Therefore this study reveals that glucose stress implications on FMD are real and significant. Whilst the serum glucose levels changed significantly during the OGTT, the macrovascular response, as represented by brachial artery FMD % levels, depressed to significantly low levels by hour 2 before recovering. The interesting finding in this study was the response of microvascular reactivity to OGTT, as represented by RVR %. These levels revealed that instead of immediate depressed reactivity to glucose load, as observed at the FMD% of macrovascular response, the values increased - albeit slightly - before showing a similar trend in reduced reactivity at the microvascular level. This unique response at the microvascular level needs to be explored.

## 5.5 Discussion

This study utilized two new validated software programmes to measure endothelial function at both microvascular and macrovascular levels. These newer systems are more reliable and measure changes instantly in an “online” fashion. Seldom have both these systems been utilized together in a simultaneous fashion. Therefore, the data obtained from this study is interesting and worth exploring further.

In the first stage, this study indicated that post-prandial hyperglycaemia manages to induce endothelial dysfunction, which could be measured both at macrovascular and microvascular levels. In addition, it was observed for the first time, that acute hyperglycaemia had an immediate but potentially divergent physiological effect at micro- and macrovascular levels of endothelial function. Macrovascular reactivity appears to be immediately and profoundly depressed with increasing glucose levels, within 1 hr of glucose intake. At the same time, microvascular reactivity and function does not appear to be depressed immediately after glucose intake. Instead, microvascular reactivity measurements appear possibly to increase in line with increasing glucose before declining function. This is an interesting finding.

In a group of fasted normo-glycaemic, normotensive ND subjects, baseline measurements of arterial reactivity in response to physiological stimulus was between 3 to 4 % at both the macrovascular and microvascular levels. At the level of the brachial artery, macrovascular response, was in line with expected FMD % for a healthy population (Witte, Westerink et al. 2005). In a meta-analysis, examining normal healthy populations with low risk, very similar to this study, the mean baseline FMD parameters were around 3.5 % in the lower quartile for the total study population (Witte, Westerink et al. 2005), a level significantly higher compared with T2DM subjects (Mean 1.52%). Similar results were obtained in other studies

(Ceriello 1998; Kawagishi, Matsuyoshi et al. 1999; Ceriello, Quagliaro et al. 2004; Abd El Dayem, El Magd El Bohy et al. 2015).

This current study revealed that OGTT in healthy ND subjects broadly resulted in decreased reactivity measurements, both at the macrovascular and microvascular levels, by 2 to 3 hrs - a finding in line with previous published data (Ceriello, Taboga et al. 2002; Dorner, Garhöfer et al. 2003; Ceriello, Quagliaro et al. 2004). However, the novel finding of an acute increase in reactivity, before the reduction in the microvascular reactivity, to OGTT needs to be explored. This finding of an initial increase in the microvascular reactivity not only differs from the reactivity response at the macrovascular level, but is also at odds with another microvascular function study performed previously (Dorner, Garhöfer et al. 2003). One simple explanation for this transient increase in reactivity could be an initial increase in blood flow, secondary to acute hyperglycaemia which could, in turn, lead to increased reactivity measurements. However, at the macrovascular level, a similar increase in flow did not translate into an immediate increase in reactivity. The mechanisms for initial difference in reactivity measurements between micro- and macrovasculature functions could potentially be explained by a number of factors.

Firstly, sympathetic innervations are absent in the retinal vessels, unlike the macrovasculature (Konno, Feke et al. 1996). Therefore, the RVR could be dependent on the local autoregulatory mechanisms involving local factors including pH, pCO<sub>2</sub>, pO<sub>2</sub> and glucose (Konno, Feke et al. 1996). The local factors appear to be much more influential in the small distal vascular beds, as noticed in the microvasculature of the retina. Therefore, the initial increase in the response of the retinal vessels could well be influenced by immediate local factors. The 'local factors' theory, thus, appears to explain the initial difference in reactivity measurements of retinal vessels post-glucose load compared with the macro vascular

response. Other studies have highlighted similar findings. Luksch and colleagues (Luksch, Polak et al. 2001) have identified that, glucose exerts an additive vasodilator effect on the ocular circulation resulting in initial increased reactivity or flow. Therefore the distinctions in the structure, function and dynamics of both micro and macrovascular beds appear to be important considerations in explaining the notable differences between them with regard to vascular reactivity.

The second potential “theory” to explain the apparent differences involves the divergence in the endothelial cells lining the vasculature. Retinal endothelium is serving an end artery when Brachial artery endothelium serves a conductance vessel. Retinal endothelial membrane is also uniquely different from endothelial lining of macrovasculature. It is part of the retinal blood/ocular barrier, which is involved in the specialized function of homeostasis of the neuro-retina (Cunha-Vaz 1979). As part of the barrier function, a layer of pericytes, which are closely related to the endothelial surface, express aldose reductase. Aldose reductase, as part of the polyol pathway of glucose metabolism, leads to endothelial dysfunction by inducing cellular damage. This ultimately leads to changes in retinopathy (Oishi, Kubo et al. 2002).

The permeability of this endothelial membrane to solutes and electrolytes is also different from any other endothelial surface (Cunha-Vaz 1979). Glucose uptake at the retinal endothelium is not dependant on insulin levels and glucose appears to move freely into the cells. Glucose uptake into the retinal endothelial cells also occurs through carrier mediated facilitated diffusion using specific plasma membrane glycol proteins, the glucose transporters (GLUT 1 and 3). These transporters are distinctive in their kinetic properties, tissue distribution and response to stimuli (Rajah, Olson et al. 2001) and regulate glucose uptake in a significantly different way to other endothelial surfaces. High glucose levels in the plasma do not down-regulate the expression of GLUT 1, as seen in other regions. Conversely, in the

retinal endothelial surface, these levels increase (Rajah, Olson et al. 2001). An acute increase in glucose levels in the retina quickly builds up sorbitol and fructose through the polyol pathway, which ultimately results in high retinal lactate levels (Kohner, Hamilton et al. 1975; Van den Enden, Nyengaard et al. 1995). The resultant acidic environment, and fall in pH accompanying hypoxia, leads to an increase in blood flow and therefore reactivity. This might explain the transient increase in reactivity seen here in the microvascular response to glucose.

The third explanation for the difference in reactivity could be based around blood flow and effect of Neuro-vascular network. Blood flow tends to increase following the release of cuff compression at the macrovascular level. Similarly, at the microvascular level, after flicker stimulation, blood flow in the retina increases (Bill and Sperber 1990; Harris, Ciulla et al. 1998). The increased blood flow in the distal micro-circulation could be leading to increased RVR before changes of acute hyperglycaemia lead to profound depression. It is unclear, at this stage, the effect of the dynamics of blood flow in two dissimilarly sized vessels with distinct haemodynamic influences and properties on the difference in reactivity between micro and macro-circulation.

After the initial difference in reactivity, both vessels revealed profound depression of reactivity and function in response to the OGTT. These findings were supported by previous studies (Kawano, Motoyama et al. 1999; Ceriello, Assaloni et al. 2005), whilst other studies (Ceriello 1998) have described other mechanism via which acute hyperglycaemia may exert its depressive effect on the endothelium. Hyperglycaemia resulted in labile non-enzymatic glycation, inducing free radical formation and oxidative stress (Ceriello, Quatraro et al. 1992). The production of free radicals during an acute rise of blood glucose concentration may occur, not only by the process of labile glycation (Ceriello, Quatraro et al. 1992), but also directly through a mechanism of auto-oxidation (Wolff and Dean 1987). Oxidative stress



is considered to represent a prime force for endothelial dysfunction. All these changes, paradoxically, appear to amplify the deleterious effects of hyperglycaemia on the retinal endothelial surface more so than the other endothelial surfaces.

In summary, for the first time, the present study describes a simultaneous but varying effect of acute glucose uptake on micro- and macrovascular endothelial function in ND subjects. It reaffirms the previously known view of acute endothelial depression on macrovascular function as a result of glucose challenge, but revealed that the blood flow dynamics of the micro-circulation responds differently. Microvascular reactivity appeared, initially, to increase secondary to flow dynamics before revealing a depressive effect of acute hyperglycaemia. This study, therefore, proposes that an acute increase in serum glucose is deleterious to both vascular beds and that this effect can be reliably detected at both sites by the newer vascular software and “online” analysis.

## **Chapter 6**

**Assessment of ED using Non invasive vascular function in high risk subjects (T2DM) compared with Healthy subjects**

## 6.1 Introduction

Current evidence supports the concept that hyperglycemia significantly contributes to the development of both cardiovascular and microvascular complications of Type 2 diabetes mellitus (T2DM) (Holman, Paul et al. 2008). Diabetic retinopathy is a highly specific microvascular complication of diabetes and the leading cause of blindness in industrialized countries (Simo and Hernandez 2009). Endothelial dysfunction is considered a key factor in the development of both cardiovascular disease (CVD) and retinopathy (Pemp and Schmetterer 2008; Xu and Zou 2009). However, whilst it is widely accepted that this condition produces a reduced circulation at the level of large arteries (Xu and Zou 2009), the data, regarding the retinal circulation, are still controversial. In patients with T2DM with absent or mild diabetic retinopathy, increased retinal blood flow has been reported (Pemp and Schmetterer 2008). Grunwald and colleagues (Grunwald, Riva et al. 1995) investigated retinal haemodynamics in the macular microcirculation, using the blue field simulation technique, and in the major retinal veins, by a combination of bi-directional laser doppler velocimetry and monochromatic fundus photography. Progression of diabetic retinopathy was assessed from fundus photographs taken at baseline and at the end of the study. The results suggested that increased flow in the macular microcirculation may be associated with progression of retinopathy, thus supporting the hypothesis that increased blood flow may play a role in the development of diabetic microangiopathy. It has been widely reported that an acute increase of glycaemia produces endothelial dysfunction at the macrovascular level in both normal subjects and patients with T2DM (Ceriello 2008). The data on the effect of acute hyperglycaemia on retinal blood flow are still controversial. It has been reported that acute hyperglycaemia does not affect retinal circulation (Gilmore, Hudson et al. 2007) or reduce it (Dorner, Garhöfer et al. 2003), and that postprandial hyperglycaemia reduces oscillatory potentials in subjects with T2DM (Arlotte, Perrott et al. 2004). Retinal function can be

measured by electro-retinogram. With this methodology, the retinal oscillatory potential is evaluated by two waves: the a-wave, used as an index of outer retinal function; and the b-wave, as an index of inner retinal function. The abnormalities in the oscillatory potentials seem to reflect severe disturbances in the retinal circulation. A correlation appears to exist between severely reduced oscillatory potentials and a circulatory deficiency in the retina (Arlotte, Perrott et al. 2004). The aim of this study was to explore the effect of acute hyperglycaemia during an oral glucose tolerance test, simultaneously at the level of macrocirculation (FMD- Flow mediated dilatation of brachial artery ) and micro-circulation (RVR- Retinal vessel reactivity of retinal artery) in subjects with T2DM.

## **6.2 Subjects and methods**

Twenty-one subjects with T2DM (duration of disease less than 7 years, 17 patients on oral agents and diet and four on diet only) and 10 healthy control subjects (previous chapter), matched for age and BMI, were recruited into the study. The clinical characteristics of the group with diabetes are reported in Table 1. All subjects were non-smokers. They had a normal resting electrocardiogram, renal function tests and blood pressure (BP). They had no history of any vascular disease or retinopathy and were not taking aspirin, statins or supplemental vitamins at the time of recruitment into the study, as these drugs can influence endothelial function (Drexler and Hornig 1999; Ceriello, Taboga et al. 2002). All subjects then had their height, weight, abdominal circumference and BMI measurements taken using the same standard equipment. Blood samples were taken either from the right or left antecubital vein in a sitting position. BP was checked with a BP monitor on the left arm and rechecked after 2 min. Finally a 12-lead electrocardiogram was performed, using the same machine for the duration of the study. Ethical approval was obtained for the conduct of the study from the Coventry Research Ethics committee, UK.

	T2DM subjects
Sex male/ female	12 / 9
Age (years)	46.4 $\pm$ 2.1
BMI (Kg/m <sup>2</sup> )	30.1 $\pm$ 1.1
Fasting plasma glucose (mmol/l)	7.8 $\pm$ 0.4
Glycated Heamoglobin HbA1C ( %)	7.8 $\pm$ 0.3
Diastolic blood pressure-resting (mmHg)	76.1 $\pm$ 1.7
Total cholesterol (mmol/litre)	4.5 $\pm$ 0.2
HDL cholesterol (mmol/litre)	1.2 $\pm$ 0.1
LDL cholesterol (mmol/litre)	2.5 $\pm$ 0.2
TG (mmol/litre)	1.6 $\pm$ 0.1
FMD (%)	2.5 $\pm$ 0.4
RVR (%)	1.4 $\pm$ 0.4

Table 6.2 Baseline characteristics of patients with T2DM

Data are expressed as means  $\pm$  SEM (standard error of mean).

FMD, flow-mediated dilatation; RVR- retinal vessel reactivity.

### 6.2.1 Study protocol

In patients with T2DM, vascular function tests, including flow-mediated dilatation (FMD) at the level of the brachial artery and retinal vessel reactivity (RVR), were performed under basal fasting conditions. At timed intervals before and after a standard oral glucose tolerance test (OGTT), both FMD and RVR were evaluated. Blood samples for assessing glycaemia were drawn at 0 (fasting state), 1, 2, 3 and 4 hr post-OGTT (75 g glucose).

### 6.2.2 Flow-mediated dilatation (FMD)

In brief, endothelial function at the macrovascular level was evaluated by measuring FMD of the brachial artery as described before. Brachial artery FMD (LOGIQ e<sub>+</sub> system; GE Vingmed Horten, NO, USA) was determined using a protocol similar to published studies (Sidhu, Newey et al. 2002). At the end of the study each day, 250 µg of sublingual glyceryl trinitrate (GTN) was administered in order to assess endothelium independent vasodilatation (Ceriello, Taboga et al. 2002). The intra-observer variability for repeated measurements of resting arterial diameter was  $0.02 \pm 0.02$  mm.

### 6.2.3 Retinal vessel reactivity (RVR) measurements

In brief, RVR (Nagel and Vilser 2004) was measured using a retinal vessel analyser (RVA; Imedos GmbH, Jena, Germany) (Seifertl and Vilser 2002) as described previously. After obtaining a clear fundus image with good contrast and no reflections, the scanning was started, according to a fixed protocol (Gugleta, Zawinka et al. 2006), as described in detail in chapter 2. The maximum vasomotion in response to flicker stimulation was measured in two

chosen segments of the major temporal inferior branch of the arteriole and venule. Three points of maximum dilation were obtained in all measuring sites (proximal and distal in arterioles and venules) and expressed as a percentage of the corresponding first baseline value.

#### 6.2.4 Biochemical and physical parameters

Blood samples were drawn at 0 (fasting state), 1, 2, 3 and 4 hr for the glucose assay, using the glucose oxidase method.

#### 6.2.5 Power calculations

To ensure that the study design had a reasonably high statistical power to detect the smallest difference in group means for FMD, the power calculation was performed in G\*Power 3.0.5 ([http://download.cnet.com/G-Power/3000-2054\\_4-10647044.html](http://download.cnet.com/G-Power/3000-2054_4-10647044.html)). The acceptable power was set conventionally to 0.80 ( $P = 0.20$ ) and statistical significance level ( $P$ ) was 0.05 (two-tailed). As no dynamic data were available for RVR, the sample size was calculated for FMD based on our previous study (Ceriello, Taboga et al. 2002). The estimated power was 0.92, revealing that the sample size of 20 subjects was adequate to determine whether the response of endothelium to glucose, during an OGTT, is significant or not.

#### 6.2.6 Validity measurements of vascular tests

All the above values were confirmed by the high levels of validity measurements recorded for each scan. Using the FMD software, the obtained values were corroborated by the low levels



of noise recorded for each scan. According to the manufacturer of the software program, a noise level of less than 40% is considered as valid for the results obtained (Sidhu, Newey et al. 2002). This confirms the validity of the values obtained at each time point for the study. Similarly, according to the manufacturer of the RVA software program, a validity measurement of more than 40% (valid cycles) was considered to be a reliable indicator of the accuracy of the results obtained (Seifertl and Vilser 2002). They were explained in detail in chapter 2.

#### 6.2.7 Statistical analysis

Statistical analysis for this study was performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA). Initially, the Kolmogorov–Smirnov test was used to determine whether the variables had a normal distribution. Baseline data comparisons between subjects with T2DM and ND subjects were performed using an unpaired Student’s t-test or the Wilcoxon signed rank test, where appropriate. In addition, for both FMD and RVR evaluation during the OGTT, non-parametric tests, Kruskal–Wallis one-way ANOVA and Wilcoxon signed ranks test, were used (Appendix III- 6.2.7.1-6.2.7.8). Statistical significance was defined as  $P < 0.05$ . Data are expressed as mean  $\pm$  SEM.

### 6.3 Results

As reported in Table 6.2, seven male and three female control subjects were recruited, while 12 male and nine female subjects with T2DM participated. The ages of the ND subjects and subjects with T2DM were  $39.8 \pm 3.6$  and  $46.4 \pm 2.1$  years, respectively, and the BMI measurements for the ND subjects and the subjects with T2DM were  $25.4 \pm 1.0$  and  $30.1 \pm 1.1$  kg/m<sup>2</sup>, respectively. Mean age for T2DM subjects was slightly older compared to ND subjects. Baseline FMD and RVR were significantly lower in subjects with T2DM compared with the ND subjects (Table 6.2).

#### 6.3.1 Serum Triglycerides (TG)

The findings below in Table 6.3.1 indicate that, with an OGTT, there is no statistically significant change to TG levels compared with baseline on subjects with T2DM subjects.

Glucose stress ND	Mean serum TG	Std. Deviation
Fasting	1.56	0.73
1 hr. post	1.57	0.71
2 hr. post	1.60	0.71
3 hr. post	1.71	0.75
4 hr. post	1.69	0.76

Table 6.3.1 Serum triglyceride (TG) levels on OGTT on subjects with T2DM

It indicates that, in subjects with T2DM, pure glucose ingestion had no significant effect on plasma TG levels. The levels remained consistent during 5 hours of testing, including the fasting phase and during an extended OGTT phase. This confirmed TG levels are not influenced by glucose ingestion, which could also be interpreted as validating the glucose challenge test, in line with expectations.

### 6.3.2 Serum Glucose

As expected, during the OGTT, glycaemia in patients with diabetes rose rapidly by 3 hr and returned to basal values at the end of the study (Figure 6.3.2.1).

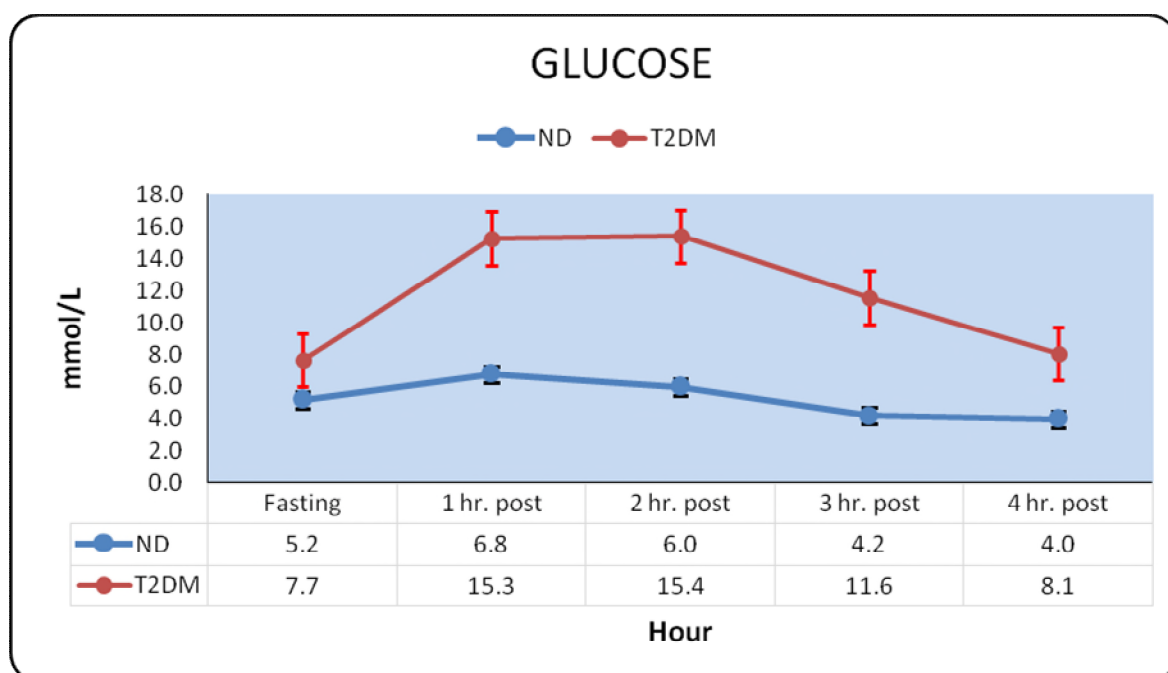


Figure 6.3.2.1 Serum Glucose levels on OGTT in T2DM subjects compared with ND (Healthy controls) from chapter 5.

### 6.3.3 Mean Flow mediated dilatation (FMD)

At 1 and 2 hr post-OGTT, the mean FMD was significantly reduced ( $P < 0.05$ ). However, at 3 hr after the OGTT, the FMD slowly started to recover and, by 4 hr post-OGTT, the FMD showed recovery. This level was comparable with the baseline level. The FMD dynamic change to the OGTT is shown in Figure 6.3.3.1 and 6.3.3.2. Endothelial independent vasodilatation was not affected in this experiment (baseline  $12.3 \pm 4.4$  vs.  $12.2 \pm 4.0\%$ ).

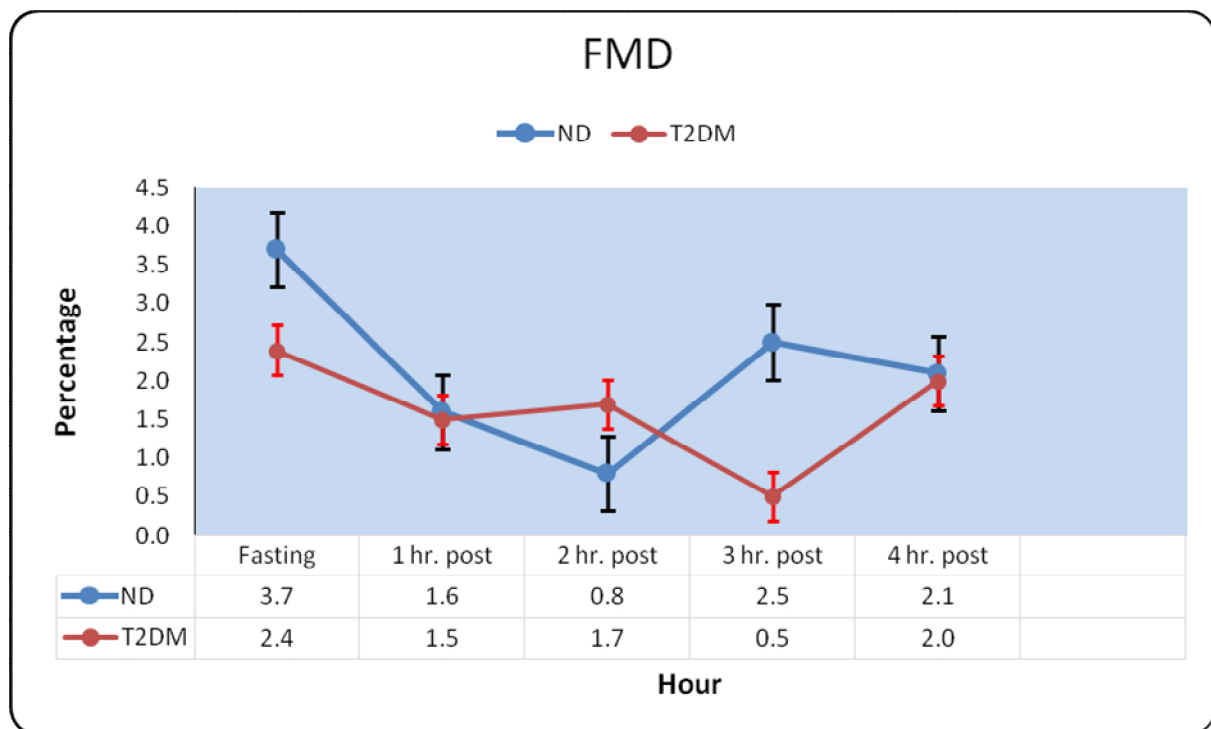


Figure 6.3.3.1 FMD % dynamic chsnge to OGTT comparing T2DM with ND (Healthy control) subjects from chapter 5.

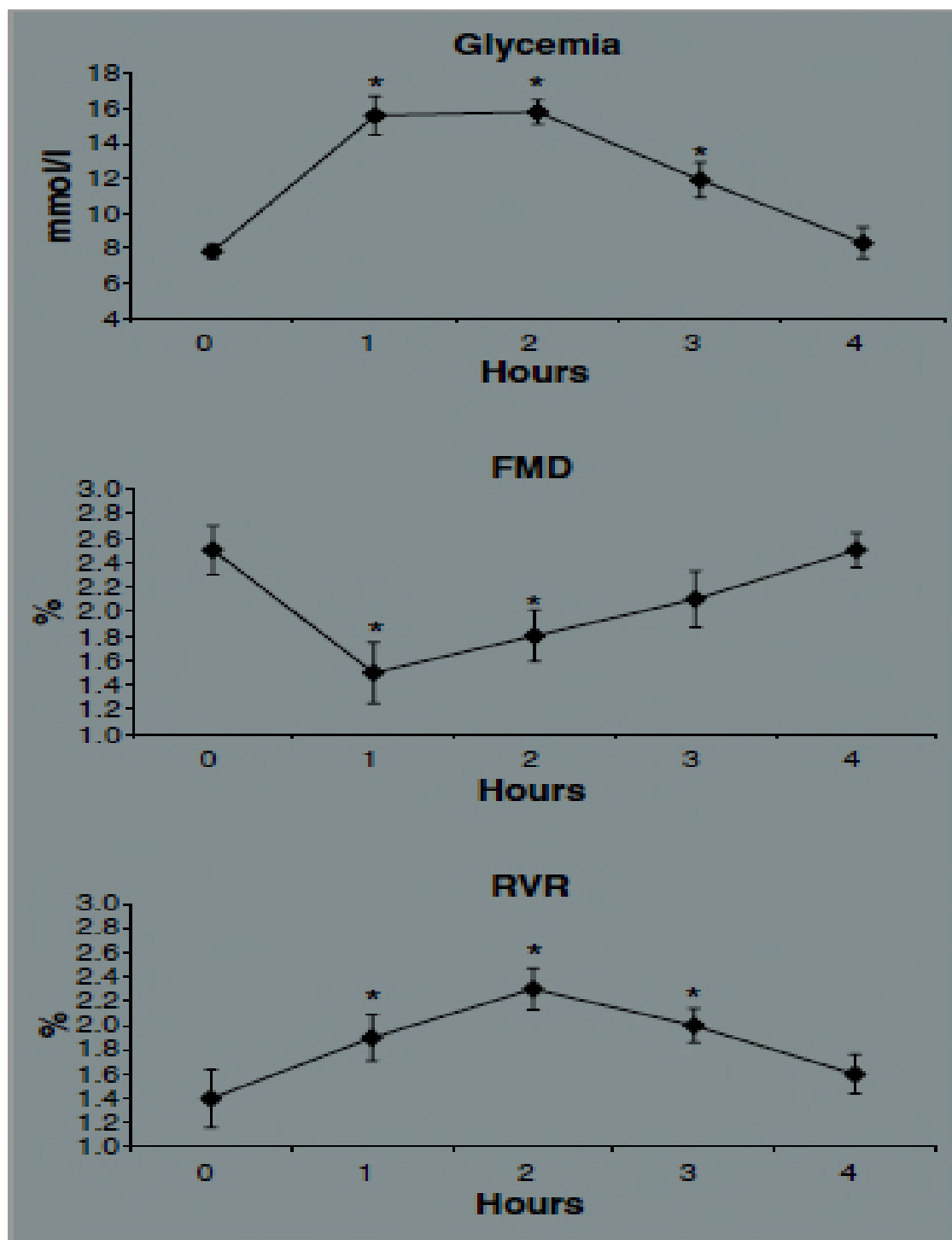


Figure 6.3.3.2 Vascular reactivity on OGTT in T2DM subjects

Glycaemia, flow-mediated dilatation (FMD) and retinal vessel reactivity (RVR) during the OGTT in subjects with T2DM. \* $P < 0.05$  vs. baseline.

#### 6.3.4 Mean Retinal vessel reactivity (RVR)

RVR was represented as percentages similar to FMD brachial artery measurements. Baseline values are calculated using the RVA software during the time when no flicker was given but the vessels were being monitored. Similarly, but in contrast to FMD, at 1 and 2 hr, and also at the 3-hr post-glucose ingestion, the mean RVR significantly increased ( $P < 0.05$ ) and was back to baseline levels at the 4 hr time point as noted in figure 6.3.3.1 and 6.3.4.1.

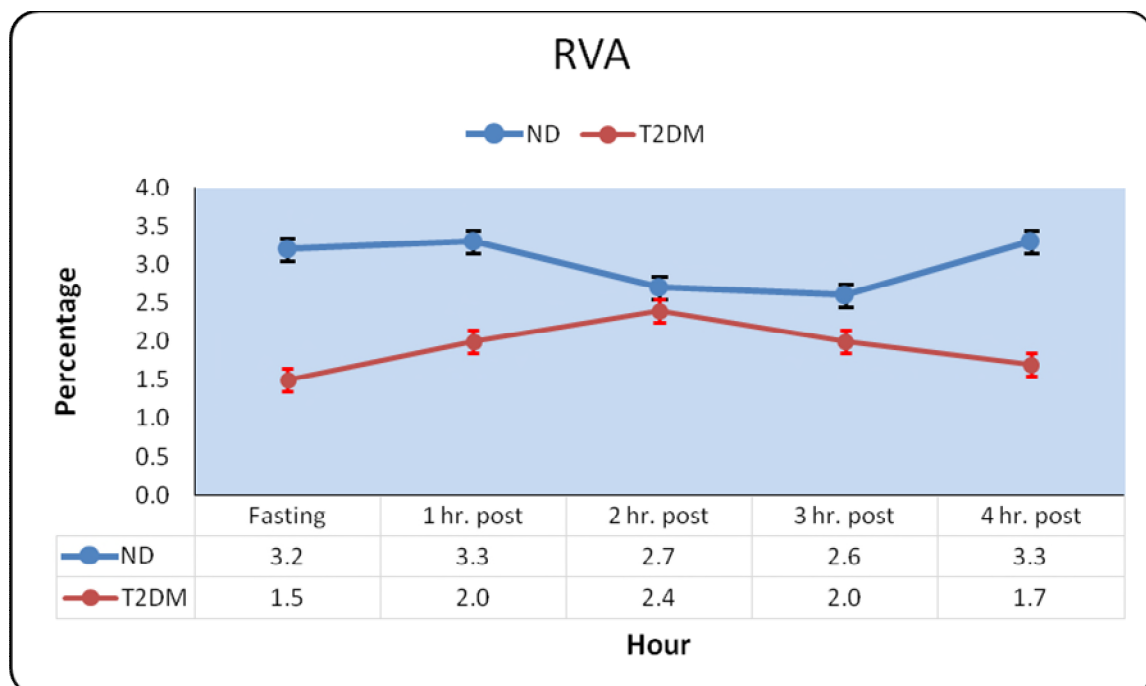


Figure 6.3.4.1 RVA % dynamic reactivity to glucose stress comparing T2DM with ND (Healthy control) subjects from chapter 5.

## 6.4 Discussion

In this study, it is reported for the first time, that an acute increase in glycaemia simultaneously induces an alteration at the level of macro-circulation of the forearm and in the retina of subjects with well-controlled T2DM as evidenced by HbA1C levels. This study further revealed that, in basal, fasting conditions, both FMD and RVR are relatively low in patients with T2DM compared with ND control subjects (from previous chapter) as noted in previous studies (Nguyen, Kawasaki et al. 2009; Pemp, Weigert et al. 2009). Whilst there is general agreement that acute hyperglycaemia induces an endothelial dysfunction at the level of the macro-circulation (Xu and Zou 2009), data on the retinal circulation are controversial. No effect or worsening action of hyperglycaemia on retinal circulation has been reported (Dorner, Garhöfer et al. 2003; Gilmore, Hudson et al. 2007).

Baseline fasting levels of endothelial function of ND subjects at both the macro and micro-vascular level, as represented by reactivity measurements, appeared to be higher compared with T2DM subjects. This phenomenon has, again, been noted in recent papers (Pemp, Garhofer et al. 2009; Abd El Dayem, Battah et al. 2014). It was clear that post-prandial hyperglycemia had a depressive effect on the endothelial function (Ceriello, Taboga et al. 2002), which could be caused via oxidative stress (Nappo, Esposito et al. 2002).

Moreover, hyperglycaemia, in the post-prandial state, appears to reduce oscillatory potentials (Arlotte, Perrott et al. 2004). It has been suggested that nitric oxide (NO) production is involved in the effect of hyperglycaemia, not only at the level of forearm circulation but also at the level of the retinal circulation (Pemp, Weigert et al. 2009). Several compounds largely used in patients with diabetes, such as ACE inhibitors, AT-1 blockers and statins, can interfere with NO action (Ceriello 2008). In other previous studies, for the most part, these factors may have not been taken into account. This study had exclusion and inclusion criteria

clearly defined to prevent any of the subjects or patients on those agents to be able to participate in the study.

Diabetic retinopathy is a highly specific microvascular complication that is a result of microvascular retinal changes ultimately leading to parenchymal changes. The two most important features of retinopathy include microvascular caliber changes and microvascular dynamic reactivity to stress, particular to physiological states such as hyperglycaemia, and people with diabetes. Apart from obvious parenchymal changes, these markers are highly significant. In a population-based prospective study, it was noted that retinal vascular caliber predicted cardiovascular death, independent of traditional cardiovascular risk factors in men and women aged 49–75 years (Wang, Liew et al. 2006). Furthermore, generalized narrowing of the retinal arterioles has now been associated with incident stroke, coronary artery disease (CAD) and hypertension, independent of other risk factors (Wang, Wong et al. 2008). The evidence that acute hyperglycaemia, during an OGTT, induces simultaneously an endothelial dysfunction in the forearm and vasodilatation in the retina is a novel finding. Endothelial dysfunction is considered an independent risk factor for a future cardiovascular events (Xu and Zou 2009), while increased blood flow in the retina is considered a risk factor for the development of retinopathy (Pemp and Schmetterer 2008). Post-prandial hyperglycaemia is an important contributing factor in the development of CVD in people with T2DM. There is now a strong clinical association between retinopathy and CVD in diabetes, with retinopathy associated with an approximate two fold increased risk for CVD and all-cause mortality in individuals with T2DM (Juutilainen, Lehto et al. 2007).

Post-prandial hyperglycaemia is now being considered an independent risk factor for the development of retinopathy in diabetes (Shiraiwa, Kaneto et al. 2005). Similarly, it was recently demonstrated that ‘post-challenge’ glucose levels measured during an OGTT might



be used as a predictor of 'postprandial hyperglycaemia'(Meier, Baller et al. 2009). With this backdrop, this study points to the finding that the macro-circulation and the retinal circulation simultaneously respond to an acute increase in glycaemia. It should also be noted that, during an OGTT, apart from glucose, changes in the levels of other hormones and insulin could be involved in endothelial influence. However, these effects should be similar in both groups and therefore unlikely to explain the disparity. This finding, therefore, has the potential to advance current opinion and help to better explain the relationship between increased CVD risk and changes of retinopathy.

## **Chapter 7**

**Effect of Metformin in reducing the Cardiovascular risk in subjects with T2DM**

## 7.1 Introduction

Cardiovascular disease (CVD) is the major cause of premature death in T2DM. T2DM is associated with a two-fold to three-fold increased risk of coronary heart disease (CHD) in men and a three-fold to five-fold increased risk in women in comparison to the non-diabetic (ND) population (Grant 2003). T2DM along with metabolic syndrome further increases the CVD risk (Athyros and Mikhailidis 2015). In diabetic CVD population, dyslipidaemia is a key feature (Ali, Jamil et al. 2015), where small dense LDL particles propagate major atherogenic events. The underlying mechanism producing small, dense LDL particles is related to hepatic oversecretion of apolipoprotein B100 (apoB100) and impaired clearance of LDL by the high-affinity LDL receptor (Taskinen 2003). The normal circulation/residence time of LDL in plasma is 3 days but this is increased up to 5 days for small, dense, highly atherogenic LDL (Packard 2003). Atherogenicity and plasma residence time of LDL may be influenced by damage to apoB100 by glycation, oxidation and nitration reactions. The quantitative amounts of such damage in healthy human subjects and diabetic patients remain unclear.

Glycation of proteins is a complex series of parallel and sequential reactions, collectively called the Maillard reaction. In the early stage, reactions of the amino acid residues are directed at the lysine and amino-terminal residues leading to the formation of early glycation adducts - fructosyl-lysine (FL), and other fructosamine derivatives. Later stage reactions lead to advanced glycation end products (AGEs). FL slowly degrades to form AGEs. Glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG) are physiological dicarbonyl metabolites and potent glycating agents, formed by the degradation of glycolytic intermediates, glycated proteins and lipid peroxidation. They react with proteins to form AGEs, directed mainly at arginine residues. The most important AGEs quantitatively are hydroimidazolones derived

from arginine residues. These are modified by glyoxal, methylglyoxal and 3-DG to G-H1 (glyoxal derivative), MG-H1(methylglyoxal derivative), and 3DG-H(deoxyglucosone derivative), respectively.

N-carboxymethyl-arginine (CMA) is a further arginine-derived adduct formed by glyoxal. Other important and widely studied AGEs are carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), and pentosidine. In addition to this, one of the markers of oxidative damage to proteins is methionine sulfoxide (MetSO), formed by the oxidation of methionine, and dityrosine.. A widely studied marker of nitration damage to proteins is 3-nitrotyrosine (3-NT) (Gaut, Byun et al. 2002).

In current clinical practice, metformin is the most widely prescribed oral glucose-lowering agent for the treatment of T2DM. It improves glycaemic control and decreases the risk of CVD (Bailey 2008; Fung, Wan et al. 2015). Ohira and colleagues have shown that metformin therapy in patients with T2DM increased the LDL particle size (Ohira, Miyashita et al. 2007) and decreased plasma concentrations of remnant lipoprotein cholesterol. Remnant lipoprotein cholesterol is a predictor of myocardial infarction (MI) and thought to reflect increased residence time and atherogenicity of cholesterol ester-rich chylomicrons and VLDL (Jialal and Devaraj 2002). Metformin also decreased the plasma concentrations of methylglyoxal in T2DM patients (Beisswenger, Howell et al. 1999) and may decrease oxidative stress (Cahova, Palenickova et al. 2015) and related oxidation of LDL (Formoso, De Filippis et al. 2008).

Therefore the aim of this chapter was to assess and quantify the lipoprotein damage in T2DM patients compared with healthy controls. The effect of metformin will then be investigated with regard to its effects on lipoprotein damage. The study would utilise the gold standard method of stable isotopic dilution analysis, “liquid chromatography– tandem mass

spectrometry” (LC-MS/MS) to measure protein glycation, oxidation and nitration adducts in apoB100 of LDL. This would help to assess the potential increased lipoprotein damage in patients with T2DM with respect to normal healthy subjects (ND) and to investigate the effect of metformin therapy.

## **7.2 Subjects and Methods**

### **7.2.1 Study population**

Patients with T2DM and healthy ND) subjects were recruited to the study from patients attending the Foleshill Medical centre, Coventry and from the Diabetes Clinics at Colchester General Hospital, Colchester, U.K. Healthy volunteers, as controls, were recruited from partners and friends of the patients with diabetes and those of investigators. Ethical approval for the study was given by the local ethics committees (Coventry Research Ethics Committee, Coventry, U.K. and North and Mid-Essex Local Research Ethics Committee, Chelmsford, U.K.). Inclusion criteria were T2DM with normoalbuminuria (albumin excretion rate 30mg/24 hr), age 40–80 years, and stable HbA1C. Exclusion criteria were individuals with significant co-morbidities who participated in any interventional study within 30 days, recipients of renal and/or pancreatic transplants and women who were pregnant or breastfeeding or of child-bearing potential not using adequate contraceptive precautions. Metformin therapy was given in the dose range 0.85–3g/day; median 1.50 g/day. The duration of metformin therapy was in the range 1–20 years; median 4 years. Participant characteristics are shown in table 7.2.1. Venous blood samples (fasting) were taken after informed consent. Plasma was separated immediately and stored at -80°C until analysis.

Study Group	Healthy subjects	T2DM subjects	T2DM subjects not receiving Metformin therapy	T2DM subjects receiving Metformin therapy
n	21	32	19	13
Age (years)	56.5 ± 9.7	60.5 ± 12.2	64.1 ± 12.8*	55.2 ± 9.5
Sex male/ female	10 / 11	14 / 18	11 / 8	3 / 10
BMI ( Kg/m <sup>2</sup> )	28.6 ± 3.6	33.7 ± 6.3‡	31.5 ± 6.3	37.1 ± 4.8‡†
Duration of T2DM (years)	--	11 ( 1-35)	13 ( 1-35)	8 ( 1-25)
Fasting plasma glucose (mmol/l)	5.14 ± 0.74	8.75 ± 2.51‡	9.15 ± 2.41‡	8.17 ± 2.62§
HbA1C ( %)	5.48 ± 0.57	8.20 ± 1.94	8.25 ± 2.04‡	8.13 ± 1.85‡
Total cholesterol (mmol/litre)	5.11 ± 1.43	4.90 ± 1.02	4.71 ± 1.00	5.18 ± 1.01
LDL cholesterol (mmol/litre)	3.18 ± 1.41	3.01 ± 1.01	2.89 ± 1.04	3.17 ± 0.98
HDL cholesterol (mmol/litre)	1.55 ± 0.81	1.24 ± 0.30	1.18 ± 0.23	1.34 ± 0.36
Triglycerides (mmol/litre)	1.16 ± 0.31	2.23 ± 1.02‡	2.13 ± 1.09§	2.37 ± 0.94‡
Systolic blood pressure (mmHg)	130 ± 20	141 ± 22	140 ± 24	142 ± 21
Diastolic blood pressure (mmHg)	77 ± 8	78 ± 8	77 ± 10	78 ± 10
GFR (Glomerular filtration rate) (ml/min)	99 ± 20	100 ± 42	89 ± 30	116 ± 52

Table 7.2.1 Characteristics of T2DM patients and healthy control subjects

Data are mean ± SD or median (minimum - maximum). Significance: \* $P < 0.05$ , § $P < 0.01$ , and ‡ $P < 0.001$ , with respect to normal healthy control subjects; and † $P < 0.05$ , with respect to T2DM patients receiving conventional therapy. Glomerular filtration rate (GFR).

### 7.2.2 Isolation of LDL

For rapid and same-day preparation of LDL, a self-generating gradient of iodixanol in a vertical rotor (S120VT) was used in a Sorvall MTX 150 micro ultra centrifuge (Hitachi). The density of plasma was increased to 12% using 60% iodixanol solution (OptiPrep; Axis-Shield). The Plasma (0.9ml) was layered under 0.9 ml of 9% iodixanol in a 2-ml ultra centrifuge tube (polyallomer, no. S302897A; Hitachi) and further void filled with 0.2ml nitrogen-purged phosphate buffered saline (PBS). The sample was centrifuged (501,000g, 16°C, 2.5 hr) with low acceleration and deceleration technique. LDL was washed with nitrogen-purged water (4ml x3) over 100-kDa microspin filters (Amicon) to remove iodixanol. The LDL was stored at -20°C until further analysis. Sample handling was performed under subdued light. Protein concentration was measured by Bradford assay. Lipid peroxidation of LDL was assessed by measuring thiobarbituric acid reactive substances (TBARS). TBARS were quantified by reference of the chromophoric response to a standard curve constructed from malondialdehyde tetramethyl acetal and expressed as malondialdehyde equivalents. The purity of LDL was assessed by “sodium dodecyl sulfate polyacrylamide gel electrophoresis” (SDS-PAGE) denaturing and agarose non-denaturing electrophoresis.

### 7.2.3 *In vitro* modification of LDL

LDL was glycated minimally by methylglyoxal and glucose *in vitro* to assess the major glycation adducts formed. LDL glycated minimally by methylglyoxal (MGmin-LDL) was prepared by incubation of methylglyoxal (200mol/l) with LDL (4.2mg/ml) in PBS (0.4 mmol/l diethylenetriamine-pentaacetic acid [DETAPAC], pH 7.4) at 37°C for 6 hr. The glycated and control LDL was washed extensively with argon-purged ice-cold water using



ultra-spin filters (Amicon 100-kDa cutoff membrane from Millipore) at 4°C, and stored at 4°C under argon and used within 2 weeks. LDL glycated minimally by glucose (AGEmin-LDL) was prepared by incubation of glucose (25mmol/l) with LDL (3mg/ml) in PBS (0.4mmol/l DETAPAC, pH 7.4) under argon (0.5ml) at 37°C for 7 days under sterile conditions. Control LDL was incubated without glucose. The glycated and control LDL were washed with argon-purged water ultrafiltration at 4°C, and stored at 4°C under argon until further analysis. Electrophoretic mobility of native and modified LDL on agarose gel electrophoresis was performed using a gel lipoprotein electrophoresis kit with barbital buffer, pH 8.6 (Helena).

#### 7.2.4 Delipidation of LDL

An aliquot of LDL solution (20µl, 100µg) was transferred into a glass tube (50 x 7.5 mm) containing butylated hydroxytoluene in methanol (5µl, 2mg/ml), 20% trichloroacetic acid (100µl), and water (75µl). The solution was mixed well, left on ice for 10 min and then centrifuged (10,000g, 15 min, 4°C). The supernatant was removed and the pellet washed with acetone (200µl) and diethyl ether (200µl) and dried under argon.

#### 7.2.5 Enzymatic digestion of apoB100

Delipidated protein was hydrolysed exhaustively by modification of a published procedure (Ahmed, Argirov et al. 2002). Protein was suspended in 100 mmol/l potassium phosphate buffer, pH 7.4 (50µl). Pronase E (20µl, 2mg/ml in 10 mmol/l KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) and 10µl penicillin (50 units/ml) and streptomycin (50µg/ml) were added and the samples incubated at 37°C for 24 hr. Thereafter, 10µl each of prolidase and aminopeptidase solutions

(2mg/ml in 10mmol/l of potassium dihydrogen phosphate-KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) was added, and samples were incubated for a further 48 hr. All steps were performed under argon. A similar method was used previously to quantify the oxidative marker, 5-hydroxy-2-aminovaleric acid in apoB100 (Pietzsch, Lattke et al. 2000).

#### 7.2.6 Protein biomarker determination by LC-MS/MS

FL, AGE, oxidation and nitration markers were determined in enzymatic hydrolysates of delipidated lipoproteins by stable isotopic dilution analysis LC-MS/MS (Ahmed and Thornalley 2003).

#### 7.2.7 Statistical Analysis

Data are mean  $\pm$  SD for parametric data and median (minimum – maximum) or (lower – upper quartile) for non-parametric data. Significance of difference between means was assessed by Student t test and significance of difference between medians, by the Mann-Whitney U test. Difference of proportions was assessed using Finney contingency tables.

## 7.3 Results

### 7.3.1 Isolation of LDL and glycation by methylglyoxal and glucose *in vitro*

A new rapid method for isolation of LDL was developed and used in this study. This method used a single ultracentrifugation step for 2.5 hr only with samples at 16°C. High purity was confirmed by a single protein band in denaturing SDS-PAGE and agarose non-denaturing electrophoresis. Lipid peroxidation of isolated LDL from normal healthy control subjects, as judged by TBARS content, was low:  $0.81 \pm 0.45$  nmol/mg protein (n=12). However, LDL glycated minimally by methylglyoxal and glucose showed increased levels of AGE residues. For glycation by methylglyoxal, MGmin-LDL showed increased content of MG-H1, CEL, and Methylglyoxal-derived lysine dimer (MOLD) residues. The major AGE formed by glycation with methylglyoxal was MG-H1 (98.4%), with minor formation of CEL (1.4%) and MOLD (0.2%). In comparison, glycation of LDL by glucose, the major increase in glycation adducts was that of FL residues with a minor increase in CML residues (Table 7.3.1).

Glycation adduct	Control 1	MGmin-LDL	Control 2	AGEmin-LDL
FL	2.21± 0.21	1.99± 0.36	3.10± 0.67	6.08± 0.37*
CML	0.031± 0.004	0.032 ± 0.007	0.056 ± 0.009	0.070 ± 0.003†
CEL	0.004± 0.001	0.024 ± 0.003*	0.011 ± 0.003	0.013± 0.003
MG-H1	0.15± 0.02	1.57 ± 0.37*	0.23 ± 0.03	0.21 ± 0.01
MOLD	0.0002± 0.0001	0.0025± 0.0009*	0.0057± 0.001	0.0092±0.002

Table 7.3.1 Changes in protein glycation adduct residues in human LDL minimally modified by methylglyoxal and glucose *in vitro*

Data are mol adduct/mol apoB100; mean ± SD (n .3). Control 1 and control 2 are incubations of LDL for 6 hr and 7 days without methylglyoxal and glucose, respectively. Significance: \* $P < 0.001$ . † $P < 0.01$ . Other adduct residues, G-H1, 3DG-H, CMA, pentosidine, MetSO, dityrosine, and 3-NT, were not changed significantly during the incubation with glycating agents.

### 7.3.2 Protein damage markers in apolipoprotein B100 of LDL in healthy human subjects and patients with T2DM

In healthy human subject's apolipoprotein B100 of LDL, the mean FL residue content was 2,900pmol/mg apoB100, equivalent to 1.49mol/mol apoB100 or 4.17mmol/mol Lys. Whilst major AGE residues quantitatively were MG-H1, median content of 46.8pmol/mg apoB100, equivalent to 0.024mol/mol apoB100 or 0.16mmol/mol Arg; and CML, median content 24.0pmol/mg apoB100, equivalent to 0.012mol/mol apoB100 or 0.035mmol/mol Lys. Median total arginine-derived AGE residue content (G-H1+MG-H1+3DG-H+CMA+

pentosidine) was 103pmol/mg apoB100, equivalent to 0.062mol/mol or 0.42mmol/mol Arg. Median total lysine-derived AGE residue content (CML+ CEL+ MOLD+ pentosidine) was 33pmol/mg apoB100, equivalent to 0.017mol/mol or 0.047mmol/mol Lys. The major oxidative marker was MetSO residues with mean content of 2084pmol/mg apoB100, equivalent to 1.07mol/mol apoB100 or 13.7mmol/mol Met. The nitration marker 3-NT had a median residue content of 2.3pmol/mg apoB100, equivalent to 0.0012mol/mol apoB100 or 0.0078mmol/mol Tyr (Table 7.3.2).

Type of Analyte	Healthy subjects	T2DM subjects	T2DM subjects not receiving Metformin therapy	T2DM subjects receiving Metformin therapy
n	21	32	19	13
Fructosamine				
FL	2900 ± 1402	3347 ± 1914	3789 ± 1971	2682 ± 1688
AGE				
CML	24.0 ( 0.7-143.7)	20.6(1.7-58.9)	20.5 (3.9-58.9)	24.1(1.7-56.4)
CEL	3.5 ( 0.2-38.9)	17.3 (3.5-59.6)*	21.9 (3.5-59.6)*	14.6(4.6-33.4) †
G-H1	3.6 ( 0.1-50.4)	31.5 (1.2-59.6)	44.0 (1.8-188.3)*	25.0 ( 1.2-59.0)†‡
MG-H1‡	46.8 (15.9-219.4)	197.0(3.0-474.4)*	235.8(45.5-474.4)*	91.3 (3.0-309.4)§
3 DG-H1	19.4 (2.2-138.9)	60.0 (4.8-163.8)*	82.3 (4.8-163.8)*	39.4 (6.3-86.2)‡∞
CMA	20.3 (0.4-47.9)	26.8 (0.7-112.8)	38.3 (0.7-112.8)†	8.9 (1.6-74.6)‡
MOLD	1.8 ( 0.3-51.5)	9.0 (0.2-31.8)∞	12.2 (0.2-31.8)∞	7.6 (1.5-27.3) †
Pentosidine	0.26 (0.03-0.84)	0.76 (0.08-2.13)*	0.75 (0.18-2.13)*	0.76 (0.08-1.61)∞
Oxidation				
MetSO	2084 ± 1360	4738 ± 3367*	5633 ± 3837*	3857 ± 2641‡
Dityrosine	0.26 (0.05-6.86)	16.7 (0.2-68.4)*	16.8 (5.8-34.8)*	11.0 (0.2-47.1)*
Nitration				
3-NT	2.3 (0.3-49.1)	0.9 (0.1-24.1)†	0.7 (0.1-15.4)∞	2.0 (0.1-24.1)‡

Table 7.3.2 Markers of protein damage in apolipoprotein B100 of LDL

Data are pmol/mg apoB100; mean ±SD or median (minimum- maximum). Significance: † $P < 0.05$ , ∞  $P < 0.01$ , and \* $P < 0.001$ , with respect to healthy control subjects; and ‡ $P < 0.05$  and § $P < 0.01$ , with respect to T2DM patients not receiving metformin therapy.

Considering all T2DM patients were studied, the mean fasting plasma glucose (FPG) concentration was increased by 70% and glycated haemoglobin was increased by 2.7% of total haemoglobin with respect to control subjects (Table 7.3.2). FL residue content of apoB100 of LDL was not increased significantly. Marked increases for contents of dicarbonyl-derived AGE residues was seen: CEL five-fold, G-H1 nine-fold, MG-H1 four-fold, 3DG-H three-fold, MOLD five-fold, and pentosidine three-fold. Median total arginine-derived AGE residue content was increased more than three-fold, 316 vs. 103 pmol/mg apoB100 ( $P < 0.001$ ); whereas total lysine-derived AGE residue content was increased only 47%, 49 vs. 33 pmol/mg apoB100 ( $P < 0.05$ ). For oxidative markers, MetSO residue content of apoB100 of T2DM patients was increased two-fold and dityrosine residue content was increased 64-fold. 3-NT residue content of apoB100 was decreased 61% in T2DM patients (Table 7.3.2). Only one protein damage marker of apoB100 in T2DM patients was linked to donor sex: median CML content was 13.3 pmol/mg for males and 30.3 for females ( $P < 0.05$ ).

### 7.3.3 Correlation analysis for markers of glycaemic control and protein damage in plasma apoB100 of T2DM patients

There was no correlation between protein damage markers of apoB100 and patient age, suggesting that the significant age difference of T2DM patients with and without metformin therapy did not compromise protein damage markers in these study groups. For markers of glycaemic control, FPG concentration correlated positively with A1C and also with FL residue content of apoB100. FL residue content correlated positively with CEL, MG-H1 and 3DG-H residue contents. There was a cluster of correlations of dicarbonyl-derived AGE residue contents: G-H1 correlated positively with MG-H1 and CMA; MG-H1 also correlated positively with CMA and 3DG-H, and also with CML and pentosidine; and 3DG-H

correlated positively with CMA. In addition, there was a cluster of correlations of oxidative marker residues with AGE residue contents: MetSO correlated positively with CEL, G-H1, MG-H1, 3DG-H, CMA, and pentosidine; and CML and MOLD correlated positively with dityrosine (Table 7.3.3). There were negative correlations of 3-NT with MG-H1 and 3DG-H.

Table 7.3.3 Correlation triangle of glycaemic control and protein damage-related variables of T2DM patients

7.3.4 Protein damage markers in apolipoprotein B100 of LDL in patients with T2DM receiving metformin.



were not significantly different (Table 7.2.1). ApoB100 of LDL from patients receiving metformin therapy had lower contents of AGEs (G-H1, MG-H1, 3DG-H, and CMA) and MetSO but higher 3-NT content than apoB100 of LDL from patients not receiving metformin therapy. MG-H1, CMA, MetSO and 3-NT residue contents of apoB100 of LDL from T2DM patients receiving metformin therapy were not significantly different from those of healthy subjects (Figure. 7.3.4).

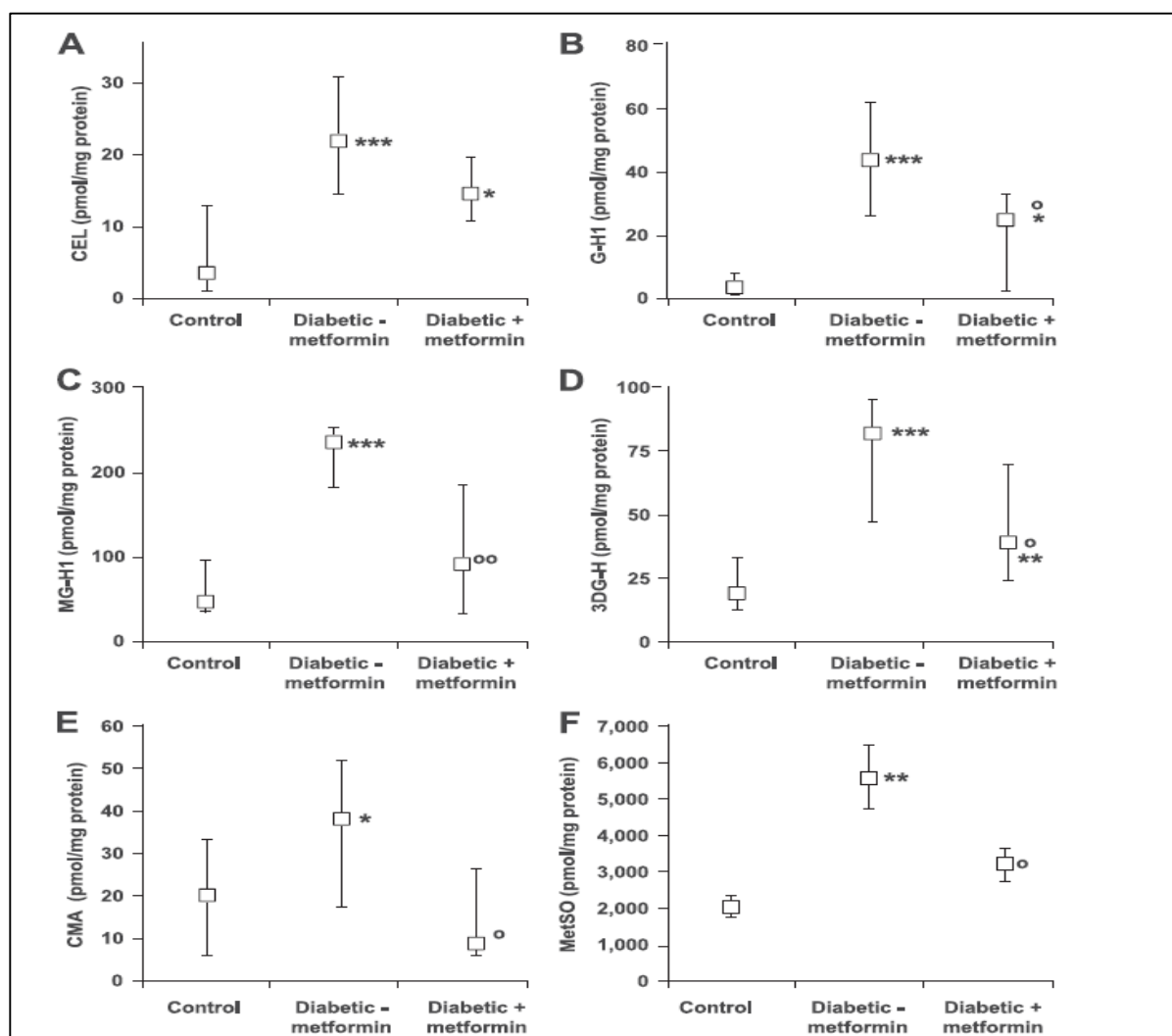


Figure 7.3.4 AGE and methionine sulfoxide residue contents of apolipoprotein B100 of LDL of T2DM patients with and without metformin therapy

(A) CEL, (B) G-H1, (C) MG-H1, (D) 3DG-H, (E) CMA, and (F) MetSO. Data are median (lower – upper quartile) except for MetSO, which is mean $\pm$ SD. Significance: \* $P < 0.05$ , \*\* $P < 0.01$ , and

\*\*\* $P < 0.001$ , with respect to normal healthy control subjects; and o $P < 0.05$  and oo $P < 0.01$ , with respect to T2DM patients not receiving metformin therapy.

## 7.4 Discussion

A new method for rapid isolation of LDL is described and used in this study. Principally, a single ultracentrifugation step of only 2.5 hr at 16°C is used during sample processing, whereas the conventional method of LDL isolation involves ultracentrifugation for 20–22 hr at 15°C (Havel, Eder et al. 1955). This rapid method has the potential to decrease the risk of apoB100 damage in pre-analytic processing and could facilitate clinical studies of LDL.

Glycation of LDL by glucose in vitro to form AGEmin-LDL showed that the major glycation adduct formed in apoB100 was FL residues with relatively minor increase of CML residue content. CML is formed by the oxidative degradation of FL. Glycation of LDL by methylglyoxal in vitro to form MGmin-LDL revealed that the major glycation adduct formed in apoB100 was MG-H1 residues (along with minor formation of CEL and MOLD residues). The rate constants for glycation of LDL by glucose and methylglyoxal are 11.2 (mol/l) / day and 28,800 (mol/l)/day respectively, at pH 7.4 and 37°C. This result is obtained based on first order glycation for both LDL and glycating agent, assuming initial rate conditions (the rate of glycation was approximately constant during the incubation time). This suggests that methylglyoxal is 2,600-fold more reactive with LDL than is glucose. The predicted in situ rates of glycation of LDL by glucose agent and methylglyoxal in plasma (McLellan, Thornalley et al. 1994; Beisswenger, Howell et al. 1999), are 73nmol/l/day and 4 nmol/l/day; suggesting that the rate of LDL glycation by glucose is 18-fold faster than by methylglyoxal in plasma. The apparent switch of relative reactivity of glucose and methylglyoxal with LDL in situ is due to the markedly lower concentration of methylglyoxal than glucose in plasma: 100nmol/l vs. 5mmol/l. The ratio of FL to methylglyoxal-derived adducts in apoB100 of healthy controls is 56. ApoB100 appears to have been glycated prior to assimilation into LDL.

The protein damage marker of highest quantitative content in apoB100 of LDL in healthy human subjects is the early glycation adduct FL, equivalent to 0.42% lysine residues. These levels are three-fold lower than reported in earlier studies using the tritiated borohydride reduction technique of 1.3% (Witztum, Mahoney et al. 1982) but similar to the 2–3nmol FL residues per milligram apoB100 estimates using the furosine technique (Schleicher, Deufel et al. 1981).

FL, MG-H1, MetSO, and 3-NT are major adducts of early glycation, advanced glycation, oxidation, and nitration of apoB100, LDL and total plasma protein. The rates of damage of LDL and plasma protein can be predicted, assuming these rates are equal to the rate of clearance of adducts in the steady state and taking into account half-lives of LDL and serum albumin, are 3 and 19 days, respectively (Langer, Strober et al. 1972). The outcome of these predictions is shown in Table 7.4.

Type of modification	Adduct	ApoB100 of LDL content (mmol/mol amino acid modified)	Plasma protein content* (mmol/mol amino acid modified)	$k_{LDL}/k_{Albumin}$	$r_{LDL}/r_{Albumin}$	LDL adduct content ( $\mu M$ )	Total plasma protein adduct content ( $\mu M$ )
Early glycation	FL	4.17	1.35	93	0.17	1.9	71.8
Advanced glycation	MG-H1	0.16	0.31	20	0.038	0.031	5.20
Oxidation	MetSO	13.7	1.93	593	1.10	1.4	9.2
Nitration	3-NT	0.0078	0.0006	943	1.76	0.0015	0.0055

Table 7.4 Comparison of the predicted reactivity of apolipoprotein B100 of LDL and total plasma protein towards early glycation, advanced glycation, oxidation and nitration

\*Data are from (Ahmed, Babaei-Jadidi et al. 2005).

$k_{LDL}/k_{Albumin}$  is the predicted ratio of the rate constants for modification of apoB100 of LDL and albumin, and  $r_{LDL}/r_{Albumin}$  is the predicted ratio of *in situ* rates of modification of apoB100 of LDL and albumin (the latter taking into account the concentrations of LDL and albumin in plasma).

Plasma concentrations of apoB100 of LDL and albumin are 1.28 $\mu$ mol/l (equivalent to 3.18mmol/l LDL cholesterol) and 682  $\mu$ mol/l (equivalent to 44mg/ml), respectively. Total plasma protein concentration is 64mg/ml, and plasma protein amino acid contents are Lys 820nmol/mg, Arg 262nmol/mg, Met 164nmol/mg and Tyr 183nmol/mg protein (Thornalley, Battah et al. 2003). All protein damage in plasma protein, other than that of apoB100 of LDL, is attributed to adduct of albumin.

Estimates of kLDL-Glucose-69 (mol/l)/day and kLDL-MG-55,452 (mol/l)/day from these deductions are not markedly dissimilar from estimates from *in vitro* glycation studies (Thornalley, Battah et al. 2003). Overestimation of rates from *in vivo* data may be attributed to glycation of apoB100 prior to assimilation in LDL particles. Overall, apoB100 of LDL is far more reactive to damage by these modifications than is albumin, even after the eight-fold greater molecular mass of apoB100 relative to albumin is taken into account. ApoB100 is highly susceptible to damage and may be a particularly good sensor of it. From the predicted *in situ* rates of modification, the rate of early and advanced glycation of LDL is only 17 and 4% of that of albumin, whereas the *in situ* rates of oxidation and nitration are 10 and 76% greater than those of albumin. As LDL has a short plasma half-life, however, the steady levels of protein glycation, oxidation, and nitration adducts in apoB100 represent only a minor part of the total plasma adduct concentration (Table 6.4). The adduct content in apoB100 of diabetic patients may be increased by effects of both increased rate of modification (due to increased plasma concentrations of modifying agents), and decreased rate of elimination.

In T2DM patients, the FL and CML residue content of apoB100 is not significantly different from that of apoB100 from healthy subjects. Increased plasma glucose concentration in T2DM patients did not produce a significant increase in FL residue content of apoB100. This may indicate that formation of FL residues in apoB100 by glucose is less favoured when the extent of glycation exceeds 2 molar equivalents, limiting further increase of FL residue content in diabetes. However, dicarbonyl-derived AGE content of apoB100 from T2DM patients is markedly increased. This suggests that dicarbonyl glycation is the main cause of increased AGE content of apoB100 of LDL in T2DM patients. Arginine-derived AGE residue contents of apoB100 in these patients increased more than three-fold.

Major quantitative oxidative damage, MetSO residue content, of apoB100 in T2DM patients is two-fold higher than in apoB100 of control subjects. This is commensurate with increased plasma peroxide concentration in T2DM patients (Banerjee, Madhusoodanan et al. 2003). There is no repair of MetSO by MetSO reductase in plasma, hence plasma MetSO likely reflects increased plasma reactive oxygen species in diabetes and decreased plasma reactive oxygen species production for patients treated with metformin.

The 64-fold increase of dityrosine residue content of apoB100 in T2DM patients, however, far exceeds this. Dityrosine residues are formed by both spontaneous and enzymatic processes. Enzymatic formation is catalysed by dual oxidase-1 (Edens, Sharling et al. 2001)—a member of the NADPH oxidase family of enzymes implicated in signaling in vascular disease in diabetes (Lambeth, Krause et al. 2008). Activation of NADPH oxidase/dual oxidase isozymes in T2DM may markedly enhance the formation of dityrosine residues in apoB100 (Bhattacharjee, Pennathur et al. 2001). Dityrosine content of apoB100 of diabetic patients (0.06mmol/mol Tyr) was intermediate between that of control subjects (0.001mmol/mol Tyr) and of apoB100 isolated from atherosclerotic plaques (0.25mmol/mol Tyr) (Leeuwenburgh, Rasmussen et al. 1997).

For T2DM patients receiving metformin therapy, there are lower contents of dicarbonyl-derived AGE and MetSO residues than in patients not receiving metformin therapy. CML residue content of apoB100 of LDL was linked to patient sex; imperfect matching of sex may have masked changes in CML residue content in patients receiving metformin therapy. Metformin decreased the concentration of methylglyoxal in T2DM patients (Beisswenger, Howell et al. 1999). It is also expected to react with glyoxal and 3-DG similarly; thereby decreasing plasma levels of these dicarbonyls and preventing related formation of AGE residues. Metformin reacts with methylglyoxal *in vivo*, forming a triazepinone adduct that has

been detected in plasma and urine (Beisswenger and Ruggiero-Lopez 2003). This decreases methylglyoxal by a scavenging action. Improvement of glycaemic control by metformin decreases dicarbonyl formation. This leads to decreased AGE formation of apoB100 indirectly, as suggested by the correlation of FL residue content of apoB100 with contents of CEL, MG-H1, and 3DG-H residues. Both mechanisms are likely to be involved.

A notable finding is the decrease in MetSO residue content of apoB100 in patients receiving metformin therapy. In correlation analysis, there are strong correlations of MetSO with G-H1, MG-H1 and CMA residue contents of apoB100. These correlations are not found in similar analysis of total plasma protein (Ahmed, Babaei-Jadidi et al. 2005), which suggests these relationships are specific to LDL. Decreased fractional clearance of apoB100 has been linked to oxidative damage of apoB100 and atherogenicity (Pietzsch, Lattke et al. 2000). Increased binding to proteoglycan in the sub-endothelium is thought to be integral to this process (Skalen, Gustafsson et al. 2002). Metformin may decrease dicarbonyl glycation of apoB100 and, in so doing, prevent decreased plasma clearance and increased oxidation and atherogenicity of LDL in T2DM. In future studies, it will be of interest to test this hypothesis in prospective placebo-controlled studies.

In this study, 3-NT residue content of apoB100 of LDL is lower in T2DM patients not receiving metformin therapy than control subjects and it normalized in patients with metformin therapy. The quantitative amount of 3-NT residues (0.03–0.1mol%) is unlikely to be damaging, but it may be a marker of NO bioavailability. Metformin therapy has been shown to be linked to activation of endothelial nitric oxide synthase (eNOS) (Calvert, Gundewar et al. 2008) and demonstrates cardioprotective micro and macrovascular effects (Bailey 2008). The changes in 3-NT residues of apoB100 of LDL here may reflect bioavailability of NO in T2DM patients, thereby suggesting that these patients receiving



metformin therapy may achieve normal vascular NO bio-availability. This suggests a further mechanism by which metformin may be protective to the vasculature in T2DM.

In summary, the new rapid method used as part of this study, to isolate LDL, could facilitate further robust clinical studies as the chances of damage to apoB100 is lesser. Finally, the work in this chapter revealed that AGE content in apoB100 of T2DM patients is higher than ND subjects. Metformin therapy appears to reduce the levels of these AGE and MetSO residues and therefore, appears to limit the damage to endothelial function, due to T2DM. Even though endothelial function was not tested in this chapter and cohort, it was demonstrated earlier on in the previous chapters that AGE products reveal a causal relationship to the development and progression of CVD (Peppas, Uribarri et al. 2002) mediated through endothelial injury and dysfunction (Wu, Liu et al. 2014).

## **Chapter 8**

### **Final discussion**

## 8.1 Discussion

Maintenance of a normal functioning endothelium is critical to prevent the onset of atherosclerosis and development of related cardiovascular complications. Endothelial function is a physiological response of the vascular endothelium to a stimulation or stress which results in a chain of activities with the release of vaso-active proteins. Under normal conditions, in healthy individuals, the endothelium performs various actions including maintaining normal vascular tone and function, limiting smooth muscle cell proliferation, maintaining homeostasis and limiting vascular inflammation (Vita and Keaney 2002; Celermajer 2005). Healthy endothelium is, therefore, essential to maintain good cardiovascular health (Poredos 2002). Impedance of this vascular control can lead to vascular dysfunction and disease which appear to be exacerbated by conditions such as obesity (Molica, Morel et al. 2015; Pareyn, Allegaert et al. 2015), hypertension, T2DM as well as hypercholesterolaemia (Vita and Keaney 2002; Endemann and Schiffrin 2004). In these conditions, the endothelial function is impaired giving rise to inflammation, thrombosis, vasoconstriction and atherosclerotic lesion formation (Levine, Keaney et al. 1995; Vita and Keaney 2002). This abnormal endothelial response, called endothelial dysfunction, is a precursor of atherosclerosis and subsequent cardiovascular disease (CVD) and events (Poredos 2002; Vita and Keaney 2002; Caballero 2003; Chan, Mancini et al. 2003) including Myocardial infarction. Whilst measuring endothelial function and dysfunction is possible at the coronary level, performing invasive measurements carries its own potential risk in leading to serious harm in rare situations (Fujino, Mikuniya et al. 1993). The kit to perform such procedures is costly and therefore could be beyond the scope for wide spread research activities at this stage. It would also be unethical to perform or even offer such studies to apparently healthy individuals at risk of future events. Therefore, developing and improving non- invasive modalities including imaging which could detect early changes reliably could

answer above difficulties. These imaging techniques could become surrogate for invasive coronary measurements and therefore predicting cardiovascular outcomes and events.

This thesis sought to utilise such non-invasive methods to measure endothelial vascular reactivity, as an indicator of endothelial dysfunction, in different risk groups and their response to various physiologic insults (Benzuly, Padgett et al. 1994; Celermajer, Sorensen et al. 1994; Heitzer, Yla-Herttuala et al. 1996; Drexler and Hornig 1999; Cai and Harrison 2000; Quyyumi 2003; Landmesser, Hornig et al. 2004; Lerman and Zeiher 2005; Deanfield, Halcox et al. 2007); (Peretz, Leotta et al. 2007). A couple of prospective studies did not reveal the expected correlation between FMD and independent future CVD prediction (Chan, Mancini et al. 2003; Fathi, Haluska et al. 2004; Frick, Suessenbacher et al. 2005). As these studies were conducted with older methods and retrospective analysis which were partly responsible for negative results, we opted for the newer method of instant online measurement for the FMD analysis.

Risk estimation for CHD on asymptomatic or low risk population is usually based on Framingham 10 year risk score (Wilson, D'Agostino et al. 1998), which estimates the risk based on traditional risk factors into low, medium or high risk. This approach has been used to initiate primary prevention measures where appropriate. However it is widely known now that CVD risk prediction is not just based on traditional known risk factors alone. Newer risk estimation algorithms include Q risk 2 and SCORE (Simmonds and Wald 2012). Even they have same drawbacks as Framingham in terms of basing the risk estimation on traditional known risk factors only. However FMD scanning and endothelial testing seem to correlate well with risk factors in predicting CVD, even in low risk populations of Framingham score (Witte, Westerink et al. 2005) and overweight children (Rodriguez, Coll et al. 2015). In fact FMD scanning has produced a high level of specificity and sensitivity in predicting coronary

disease in subjects with peripheral arterial disease. As this group of patients is at higher risk of underlying systemic vascular dysfunction, accurate prediction was very valuable. In this regard, FMD at least correlated with results obtained from myocardial perfusion scanning results, validating the potential for FMD scanning (Perrone-Filardi, Cuocolo et al. 2005).

This current thesis sought to investigate subjects with obesity including early onset, IGT, T2DM as well as non-obese, non-T2DM subjects (NOC) using physiological stress as a stimulus to evaluate microvascular and macrovascular reactivity. The technology used to assess both vascular beds was previously validated in the defined subjects groups (Polak, Dorner et al. 2000; Newey and Nassiri 2002; Seifertl and Vilser 2002; Sidhu, Newey et al. 2002).

A standardized online technology for assessing vascular function of macro-vessels was used as a surrogate to test a new technology for vascular function testing in micro-vessels, which was, to that date, not been determined in cohorts with metabolic risk. Although micro-vascular reactivity has been studied in a healthy population (Dorner, Garhöfer et al. 2003), a comparative study in healthy controls versus groups with different metabolic states has not been systematically examined before. Therefore, the work in this thesis sought to explain the phenomenon described above.

These current studies identified that microvascular reactivity assessment provided a reliable indicator of the functional status of microvasculature. The study revealed dynamic reactivity changes acutely to physiologic stress in different metabolic groups. This is an important finding as previous studies have shown that macrovascular reactivity, as assessed by FMD, may predict prognostic disease (Cai and Harrison 2000; Schächinger, Britten et al. 2000) (Heitzer, Schlinzig et al. 2001; Gokce, Keaney et al. 2002; Gokce, Keaney et al. 2003). Similarly, endothelial dysfunction at the brachial level could represent and correlate similar

dysfunction in coronary vessels (Anderson, Uehata et al. 1995; Takase, Uehata et al. 1998; Pyke and Tschakovsky 2005). Therefore, the next step would be to investigate potential for a possible link between dynamic change in microvascular reactivity and cardiovascular outcome measures.

Within this thesis, microvascular reactivity was assessed through imaging retinal vessels, by applying flickering light as stimulus before and after a meal. Previous studies have shown that flicker light can induce vasodilatation (Nagel and Vilser 2004; Gugleta, Zawinka et al. 2006) and blood flow (Michelson, Patzelt et al. 2002) of retinal vessels in a healthy population. This response appears to be attenuated with hyperglycaemia (Dorner, Garhöfer et al. 2003). It has also been shown that known subjects with T2DM have a reduced vaso-reactivity of their microvessels compared with healthy subjects (Mandecka, Dawczynski et al. 2007; Nguyen, Cheung et al. 2008; Pemp, Garhofer et al. 2009). In the first instance, this current thesis revealed that, macrovascular reactivity appears to be immediately and profoundly depressed, within 1 hr of glucose intake in healthy subjects. At the same time, microvascular reactivity and function does not appear to be depressed immediately after glucose intake. Instead, microvascular reactivity appears to initially increase in line with increasing glucose. This is an interesting finding. Following the initial difference in reactivity, both vessels revealed further depression of reactivity and function in response to the OGTT.

This study revealed that, in basal fasting conditions, both FMD and RVR measurements are low in patients with T2DM compared with healthy control subjects in line with previous studies (Nguyen, Kawasaki et al. 2009; Pemp, Weigert et al. 2009). Other risk groups including Obese and IGT subjects also appear to reveal similar trend of low reactivity measurements compared to healthy control subjects.

Further more, this study evaluated the effects of fat ingestion on reactivity measurements and correlated with markers of both endothelial and inflammatory markers for acute dynamic changes. These studies revealed that circulating endotoxin levels rose in all subjects, irrespective of their metabolic status, although circulating endotoxin revealed dramatic post-prandial change in the high metabolic risk groups. Specific comparative analysis of ND subjects versus subjects with T2DM at 4hr post-prandial, identified that the latter had a mean endotoxin level 125.4% higher than ND subjects. During the same period, RVA and FMD parameters were measured. This study revealed statistically significant correlations between increasing triglyceride (TG) levels post fat meal with impaired functioning of endothelial cells at both macrovascular and microvascular levels.

Cumulative data derived from the fasting state and the SFA post-prandial state indicated that T2DM subjects are subjected to 336% more circulating endotoxin than ND subjects over the 4hr duration. Based on these studies, it might be deduced that more frequent saturated fat exposure may exacerbate endotoxin, inflammation and depress vascular reactivity. Also, smaller more frequent meals have the potential to allow endotoxin to spike several times a day. However no direct associations were able to be noted between circulating endotoxin levels and vascular function in these cohorts.

High childhood BMI was associated with an increased incidence of adult diabetes and coronary artery disease (Llewellyn, Simmonds et al. 2015). Clinical studies have implicated gut derived endotoxin as a direct 'primary mediator' to activate the inflammatory state, contributing to metabolic disease, with current cross sectional data showing elevated systemic endotoxin levels in conditions of obesity, coronary artery disease and type 2 diabetes mellitus (Creely, McTernan et al. 2007; Piya, Harte et al. 2013; Eguchi and Manabe 2014). Endotoxin as a potential biomarker of sub-clinical inflammation was examined for early CVD risk in

childhood obesity. Our study revealed that in childhood obesity, circulating endotoxin is significantly correlated with pro-inflammatory markers, TNF- $\alpha$ , MCP-1, as well as biomarkers of atherogenesis. Endotoxin may also account for, in part, the continual pro-inflammatory state experienced in obese children (Gilardini, McTernan et al. 2006; Al-Attas, Al-Daghri et al. 2010; Kheirandish-Gozal, Peris et al. 2014; Chang, Jian et al. 2015). As we already know that, CVD development in later life shows an increased risk when the condition is preceded by prior chronic inflammatory conditions (John and Kitas 2012; Banerjee, Biggs et al. 2013; Eguchi and Manabe 2014; Olza, Aguilera et al. 2014; Kranendonk, van Herwaarden et al. 2015), this data supports that theory. This study did not look into other potential causes of high endotoxin levels arising from gut such as irritable bowel syndrome. As endotoxin levels appear to be related to type and frequency of diet, future studies could be directed to address potential diet composition and types of diet that prove beneficial and reduce endotoxin load from gut, particularly in high risk subjects like obese children.

Finally, whilst investigating the lipoprotein damage in the high risk T2DM group, it became apparent that high fat meal led to immediate increase in AGE products which have long term detrimental effects on the endothelium. The oxidative and glycative damage to LDL is less pronounced in the groups who were taking metformin as medication to control hyperglycaemia in T2DM compared with subjects not on Metformin. This study revealed that 3-NT residue content of apoB100 of LDL is lower in T2DM patients not on metformin therapy than control ND subjects and 3-NT residue is normalized in patients with metformin therapy. As 3-NT is a marker of NO bioavailability (Ceriello, Bortolotti et al. 1998; Ceriello, Taboga et al. 2002), the changes in 3-NT residues of apoB100 of LDL may reflect bioavailability of NO in T2DM patients, thereby suggesting that these patients receiving metformin therapy may achieve normal vascular NO bio-availability. Metformin therapy has also previously been shown to be linked to activation of endothelial nitric oxide synthase



(eNOS) (Calvert, Gundewar et al. 2008). This suggests a further mechanism by which metformin may be protective to the vasculature in T2DM.

Finally, CVD risk groups demonstrate varying levels of underlying chronic pro-inflammatory state which could be detected by non-invasive imaging modalities. The pro-inflammatory state is noticeable as early as childhood state with appropriate risk profile. Over time, it is hoped that these non-invasive methods could lead to pharmacological and therapeutic interventions at an early stage thereby reducing the risk of CVD and prolonging healthy life.

## **8.2 Future directions**

Endotoxin appears to be an exciting and promising marker which, in different metabolic groups- appears to vary linking relative high risk to CVD. As this study was studying changes related to acute effects of a meal, Endotoxin revealed significant acute change in differing conditions in different metabolic groups. Therefore this marker reveals promising potential for future studies linking CVD with gut related illnesses, dietary habits, type and nature of diet and even lifestyle related issues.

The UK National screening programme for diabetic patients involves yearly digital photography, mainly identifying parenchymal changes. Functional changes of arterial and Retinal vessel reactivity precedes atherosclerotic changes (Celermajer, Sorensen et al. 1992; Benzuly, Padgett et al. 1994; Landmesser, Hornig et al. 2004) by years. Therefore, it is possible that, by using this retinal function study software and video added to the current screening service, subjects with diabetes could be further risk stratified for early stage CVD. Another area of future research around this technology could be based around examining retinal vessel calibre which this study acquired the data but had no chance to analyse or

investigate further. The calibre of both retinal artery and vein could be analysed as part of routine study, information which could be used as a predictor of CVD. In a population-based prospective study examining retinal vascular calibre, calibre size of the vessels predicted cardiovascular death, independent of traditional cardiovascular risk factors in men and women aged 49–75 years (Wang, Liew et al. 2006). Generalised narrowing of the retinal arterioles has also been associated with stroke, coronary artery disease (CAD), as well as hypertension (Wong, Knudtson et al. 2004). It has also shown that narrower retinal arterioles are associated with lower hyperaemic myocardial blood flow and perfusion reserve in asymptomatic adults, concluding that retinal arteriolar narrowing may serve as a marker of coronary microvascular disease (Wang, Wong et al. 2008). Further analysis of retinal vascular calibre has predicted CHD death independent of traditional cardiovascular risk factors in men and women aged 49–75 years (Konno, Feke et al. 1996).

Newer method of measuring brachial artery reactivity using FMD as described in this thesis could be used as a screening tool to further risk stratify healthy groups – to start with- people involved in high risk sports like mountain biking, cross country running, and other high endurance sports groups and clubs.

### **8.3 Conclusion**

This thesis has sought to examine endothelial dysfunction in at-risk patients and groups using updated and new technology. The aim was to investigate whether dynamic micro and macrovascular changes can be identified acutely after induction of physiological stress. It is recognized that, early intervention at vascular level could lead to potential reduction in future cardiovascular events (Halcox, Schenke et al. 2002; Chan, Mancini et al. 2003; Fathi, Haluska et al. 2004; Celermajer 2005; Frick, Suessenbacher et al. 2005). Therefore, studying

the vascular reactivity, through the FMD and RVA, may contribute to provide the required individualized prevention strategies to reduce the burden of cardiovascular disease. Finally both these non invasive methods could be utilised not only in research arena, but also in screening areas for sports clubs, high risk sports and finally in commercial domain investigating the effects of drugs on endothelium, both immediate and long term in various metabolic groups.

## **Appendix I**

### **Methods and check lists**

**1 a Patient's information sheet**

**Participant Information Sheet**

*“Are post-prandial hyperglycemia and post-prandial hypertriglyceridemia simultaneously harmful for both macro and microcirculation in diabetes?  
A strong clinical implication”*

**Investigation in to effect of excess Glucose / sugar uptake in blood after a meal on medium and small blood vessels in healthy and Diabetic patients**

Dear sir/madam

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

**What is the purpose of the study?**

Type 2 diabetes mellitus is the consequence of both insulin resistance and impaired insulin secretion. In the progression from normal glucose tolerance to diabetes, postprandial glucose (Glucose levels in blood after a meal) levels often rise before fasting plasma glucose levels increases. This impaired glucose tolerance is associated with increased risk for blood vessel disease and can adversely affect the heart.

The mechanism by which excess glucose affects blood vessels is by way of “endothelial dysfunction”. **Endothelial dysfunction** is a physiological derangement of normal biochemical processes carried out by the cells that line the inner surface of all blood vessels including arteries and veins. Compromise of normal function of endothelial cells is characteristic of endothelial dysfunction. It is thought to be a key event in the development of “atherosclerosis”, furring up of arteries and often this becomes a fertile ground for future problems including stroke and heart attacks. A key feature of endothelial dysfunction is the inability of blood channels to dilate fully in response to stress. Postprandial hyperglycemia, in which elevated and prolonged postmeal glucose concentrations are evident, often precedes an elevation of fasting glucose level in the progression from normal glucose tolerance to impaired glucose tolerance (IGT) and subsequently diabetes.

September 11, 2007, version2

1

Madhu varma Chittari

Page 1

**Participant Information Sheet**

We aim to study the effect of excess glucose levels in blood after a meal on blood vessels both medium size in the arm and smaller vessels in the back of the eye. This will help in understanding the effect of such high levels on the blood vessel wall and help to better explain the risk of heart disease. Healthy people are studied not only to understand its effects on small and medium blood vessels in general but also to better show the difference and risk in diabetic population. This study will become part of an educational qualification.

**Why have I been chosen?**

You have been chosen either because you are a diabetic or you are matched to one. A total of up to 200 patients will be screened for the study to select 50 patients who will be enrolled for the study.

**Do I have to take part?**

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**

You will be asked to fast overnight and attend your own General Practice or other centre closer to your home for a screening visit. This involves a specialist nurse or a study doctor confirming that you would like to take part in the study. An opportunity will be given to ask any questions. After obtaining written consent, personal information including your past medical history, dietary, smoking and exercise habits and your current treatment will be taken. Simple measurements such as height, weight, tummy measurements and blood pressure will be recorded. A heart tracing will be performed. A urine sample will be requested. Finally a sample of blood (not more than 5-8 teaspoonfuls) (up to 30 mls) taken for baseline routine tests like glucose and cholesterol. All this should take 30 minutes of your time in the morning and then you will be free to leave.

We will then create a list of patients to be enrolled into the study after matching as closely as possible people with and without Diabetes. We will invite 50 patients (25 healthy and 25 diabetics) to participate in the full study.

If you are one of the 50 to be invited, then you will be asked to attend the University Hospital at Walsgrave, Coventry for 3 days of tests. Each day you will be spending about 5 hours with us in the morning at the Diabetes centre. You can choose the day within reasonable options, but all the 3 days of tests should be completed within 1 month.



On the days of tests, you will be fasting overnight for 10 hours and once at the Diabetes centre, We will take 5- 8 teaspoonfuls (up to 30 mls) of blood and we will then give you either a glucose (Sweet) drink, cream or both on 3 different days. After taking one of the above, we will repeat similar blood test at 1, 2, 3 and 4 hours duration. We will use an ultrasound scan machine to scan your arm looking for changes in the blood vessel of your arm at similar intervals. Scanning is done using a small probe with jelly and it is entirely painless.

The non invasive measurement takes 10-15 minutes.

Scanning of the eyes will be done after dilating the pupils with eye drops. The painless procedure to capture a picture of blood channels at the back of the eyes lasts 15 minutes.

You will be provided with lunch at the end of this and you are free to leave.  
Any reasonable expense towards transport and parking will be reimbursed to a maximum of £27.

**What do I have to do?**

We would like you to inform us if you are prescribed any new medications like aspirin or statin. We would also wish to know if you wanted to take any medical health product like vitamins or non prescription / herbal products.

**What are the risks/disadvantages of taking part?**

None identified at this stage as we do not give any medication. Diabetic patients and healthy volunteers are expected not to smoke, or take any non prescription medications as well as expected to avoid taking aspirin or statin tablets if not clinically necessary.

**What are the possible benefits of taking part?**

We cannot promise the study will help you but the information we get might help improve the treatment for diabetic people yourself as well as people with similar condition.

**What if there is a problem?**

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer, then normal NHS redressal mechanism applies. Alternately, you can contact Cathy Charlton, University secretary, Main campus, The University of Warwick, Coventry CV4 8UW. Phone number: 02476522713. Email: c.e.charlton@warwick.ac.uk

**Will my taking part in this study be kept confidential?**



All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.  
Your GP may be notified of your participation in the study with your consent..

**What will happen if I don't want to carry on with the study?**

You can withdraw from the study at any time and your data/samples will be destroyed immediately. This decision will not influence your treatment either with your GP or at the hospital clinics.

**What will happen with the results of the research?**

Dependent on the findings of the research the results are likely to be published in a scientific journal; this would be available for public perusal. Your specific details will not appear in the journal but the coded information collected as well as the results from all the patients involved might be presented.

**Who has reviewed the study?**

This study was reviewed by Coventry Research Ethics Committee which is entirely independent of any hospital trust.

**Who is organizing and funding the research?**

The blood samples are being collected as part of a large research programme run by Professor S. Kumar and prof. Antonio Ceriello. These studies are funded by a number of research funding bodies and charities.

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you for considering taking part or taking time to read this sheet.

Contact for further information:

**Dr Madhu Varma Chittari**  
Clinical Research Fellow  
Diabetes Centre  
Rugby St Cross Hospital  
University Hospitals Coventry and Warwickshire NHS Trust  
Rugby CV22 5PX  
Phone: 01788667743

**1 b Patient's Informed consent form**

Centre Number:  
Patient Identification Number for this trial:

### CONSENT FORM

Title of Project:

"Are post-prandial hyperglycemia and post-prandial hypertriglyceridemia simultaneously harmful for both macro and microcirculation in diabetes? A strong clinical implication"

Investigation in to effect of excess Glucose / sugar uptake in blood after a meal on medium and small blood vessels in healthy and Diabetic patients

Name of Researcher: Dr Madhu Varma Chittari / Professor Antonio Ceriello

Please initial box

- |    |   |                          |
|----|---|--------------------------|
| 1. | I confirm that I have read and understand the Information sheet dated September11, 2007 (version 2) for the above study and have had the opportunity to ask questions.  | <input type="checkbox"/> |
| 2. | I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.  | <input type="checkbox"/> |
| 3. | I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. | <input type="checkbox"/> |
| 4. | I agree to my GP being informed of my participation in the study.   | <input type="checkbox"/> |
| 5. | I agree to storage of my donated blood for future use in the research Programme   | <input type="checkbox"/> |
| 6. | I agree to take part in the above study.  | <input type="checkbox"/> |

Name of Patient \_\_\_\_\_ Date \_\_\_\_\_

Signature

Name of Person taking consent \_\_\_\_\_ Date \_\_\_\_\_  
(if different from researcher)

Signature

Researcher \_\_\_\_\_ Date \_\_\_\_\_ Signature

When completed, 1 for patient; 1 for researcher site file;  
1 (original) to be kept in medical notes. Version 2 September 2007

**1 c Patient's proforma and check list**

Appendix I      2.1.4.2 Patient's proforma and check list

1 M V Chittari Microvascular Project

**Patient No.**

**Code No.**

**Screening Visit**

**Investigation in to effect of excess Glucose / sugar uptake in blood after a meal on medium and small blood vessels in healthy and Diabetic patients**

**Dr Madhu Varma Chittari / Professor Antonio Ceriello**

**Baseline Details:**

Date of Birth		Age	
Sex	Male    1	Female    2	
Ethnicity	South Asian    1	Caucasian    2	
Weight	Height		
Abdominal circumference	cm		
BMI ( Kg/m2 )	(18.5-24.9)    1	(25-29.9)    2	(30-39.9)    3
Blood Pressure ( Sitting )		Repeat	
Diabetes Mellitus	Yes    1	No    2	
Diet control DM	Yes    1	No    2	
Urine: Micro-albuminuria	Yes 1    No 2		
Urine : Albumin / creatinine ratio			

## Appendix I 2.1.4.2 Patient's proforma and check list

2 M V Chittari Microvascular Project

Insulin	Yes	1	No	2
Family His DM	Yes	1	No	2
Duration DM	< 3 YEARS	1	> 3 YEARS	2
Smoking( current )	Yes	1	No	2
X Smoker	Yes	1	No	2
Vascular Disease ( Any )	Yes	1	No	2
Excess Alcohol Units per week	Yes	1	No	2
Aspirin	Yes	1	No	2
Statin	Yes	1	No	2
Vitamins (Any)	Yes	1	No	2
Any other non prescription Medication	Yes	1	No	2
Specify				
Consent	Fasting Bloods		12 Lead ECG	
Urine for Pregnancy test if relevant				

**M Varma Chittari**

## **1 d Validated Dietary Questionnaire**

**FOOD INTAKE**

Surname .....

Subject ID

First Name(s) .....

Address .....

Questionnaire No.

.....

Group Code

.....

Survey Number

Male / Female

Date of Birth ..... Date of Survey .....

The following questions are about the foods you USUALLY eat.  
Please indicate the number of days per week that you eat each item on average. Ring the answer as in these examples :

If you eat the food every day, ring 7      7 6 5 4 3 2 1 F R

If you eat the food three days/week, ring 3      7 6 5 4 3 2 1 F R

If you eat the food once a fortnight, ring F      7 6 5 4 3 2 1 F R

If you rarely or NEVER eat the food, ring R      7 6 5 4 3 2 1 F R

☐☐

1-8

☐

9

☐

10

☐

11-12

☐

13

☐

14-15

☐

16-17

☐

18-21

☐

22-23

☐

24-25

☐

26-29

**BREAD**

How often do you eat the following breads and how many slices do you have per day?

White bread

7 6 5 4 3 2 1 F R

☐

30

How many slices do you have per day?

.....

☐

31

What is the usual size of slice?

1. Large thick
2. Large medium
3. Large thin
4. Small thick
5. Small medium
6. Small thin

☐

32

Brown, 50/50 or wheatgerm bread

7 6 5 4 3 2 1 F R

☐

33

How many slices do you have per day?

.....

☐

34

What is the usual size of slice?

1. Large thick
2. Large medium
3. Large thin
4. Small thick
5. Small medium
6. Small thin

☐

35

Wholemeal bread or chapatis

7 6 5 4 3 2 1 F R

☐

36



How many slices/chapatis do you have per day? .....

☐ 37

What size of slice do you have?

☐ 38

1. Large thick
2. Large medium
3. Large thin
4. Small thick
5. Small medium
6. Small thin
7. Chapatis only

Other bread: rolls, teacakes, croissants, crumpets, pitta, naan

☐ 39

7 6 5 4 3 2 1 F R

How many rolls/pieces do you have per day? .....

☐ 40

What type of other bread do you have?

☐ 41

1. White roll (e.g. with burger or hotdog)
2. Brown roll
3. Wholemeal roll
4. Teacake
5. Croissant
6. Crumpet
7. Pitta bread/wrap
8. Naan bread
9. More than one of these

Crispbread, Ryvita or cream crackers 7 6 5 4 3 2 1 F R

☐ 42

How many do you have per day? .....

☐ 43

How often do you have jam/marmalade/honey on bread? 7 6 5 4 3 2 1 F R

☐ 44

## BREAKFAST CEREALS

How often do you eat the following cereals?

☐ ☐ 45-46

☐ ☐ 47-48

- |   |                   |
|---|-------------------|
| 1. Cornflakes   | 7 6 5 4 3 2 1 F R |
| 2. Frosties, Nut Cornflakes, Sugar Puffs, Ricicles, Coco Pops | 7 6 5 4 3 2 1 F R |
| 3. Rice Krispies or Special K                                 | 7 6 5 4 3 2 1 F R |
| 4. Muesli, Fruit 'n' Fibre or Cheerios                        | 7 6 5 4 3 2 1 F R |
| 5. Weetabix, Wheatflakes or Shredded Wheat                    | 7 6 5 4 3 2 1 F R |
| 6. Bran Flakes or Sultana Bran                                | 7 6 5 4 3 2 1 F R |
| 7. Porridge or Ready Brek                                     | 7 6 5 4 3 2 1 F R |
| 8. All Bran   | 7 6 5 4 3 2 1 F R |

How many teaspoons of sugar/honey do you add? .....

☐ 49

How often do you have wheat bran? 7 6 5 4 3 2 1 F R

☐ 50

**MEATS****How often do you have the following meats and meat dishes?**

Beef: roast, steak, stewed, burgers, lasagne, bolognese, chilli, curry

7 6 5 4 3 2 1 F R

☐ 51

Lamb: roast, chops, stewed, curry

7 6 5 4 3 2 1 F R

☐ 52

Pork: roast, chops, stewed, curry, sweet &amp; sour

7 6 5 4 3 2 1 F R

☐ 53

Bacon

7 6 5 4 3 2 1 F R

☐ 54

Ham or gammon (include use in composite dishes)

7 6 5 4 3 2 1 F R

☐ 55

Chicken/other poultry: roast, casserole, curry, sweet &amp; sour

7 6 5 4 3 2 1 F R

☐ 56

Canned meat (e.g. corned beef), pate or meat spread

7 6 5 4 3 2 1 F R

☐ 57

Sausages

7 6 5 4 3 2 1 F R

☐ 58

What type of sausages do you have?

1. Pork
2. Beef
3. Pork & Beef
4. Turkey
5. Low Fat
6. Frankfurters / Hot Dog

☐ 59

Meat pie/pastie, sausage roll, samosa - shop bought

7 6 5 4 3 2 1 F R

☐ 60

Meat pie/pastie, sausage roll, samosa - home made

7 6 5 4 3 2 1 F R

☐ 61

Liver, kidney or heart

7 6 5 4 3 2 1 F R

☐ 62

Do you usually eat the fat on meat?

Yes / No

☐ 63**FISH****How often do you eat the following fish?**

White fish: cod, haddock, plaice, fish fingers, fish cakes

7 6 5 4 3 2 1 F R

☐ 64

Kipper, herring, mackerel, trout (including canned)

7 6 5 4 3 2 1 F R

☐ 65

Pilchards, sardines, salmon (including canned)

7 6 5 4 3 2 1 F R

☐ 66

Tuna (including canned)

7 6 5 4 3 2 1 F R

☐ 67

Shellfish, e.g. prawns

7 6 5 4 3 2 1 F R

☐ 68**VEGETABLES & SAVOURY DISHES****How often do you have the following vegetables or dishes?**

Boiled or mashed potatoes	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 69
Jacket potatoes	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 70
Shop bought chips, 'oven chips', hash browns	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 71
Home-cooked chips	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 72
Roast potatoes	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 73
Peas	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 74
Other green vegetables, salads or tomatoes	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 75
Carrots	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 76
Parsnips, swedes, turnips or sweetcorn	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 77
Baked beans	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 78
Butter beans, broad beans or red kidney beans	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 79
Lentils, chick peas or dahl	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 80
Onions (cooked/raw/pickled)	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 81
Spaghetti, other pasta, noodles (e.g. with bolognese, tomato sauce, or in lasagne)	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 82
Rice (e.g. with curry, chilli or other savoury dish)	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 83
Quiche	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 84
Pizza	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 85
Vegetarian burgers/sausages	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 86
Dishes made with TVP (soya mince) or Quorn	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 87

### BISCUITS, CAKES & PUDDINGS

How often do you eat the following items?

Digestive biscuits/plain biscuits	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 88
Other sweet biscuits	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 89
Chocolate, e.g. Galaxy, Mars bar, Twix, Kit Kat	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 90
Sweets, e.g. fruit gums, pastilles, mints	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 91
Crisps/savoury snacks, e.g. Quavers, tortilla chips	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 92
Nuts	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 93



Ice cream, iced dessert, fool, mousse or trifle	7 6 5 4 3 2 1 F R
Low fat yogurt	7 6 5 4 3 2 1 F R
Low calorie yogurt, e.g. Shape	7 6 5 4 3 2 1 F R
Other yogurts/fromage frais, e.g. thick & creamy	7 6 5 4 3 2 1 F R
Fruit cake/sponge cake/sponge pudding - shop bought	7 6 5 4 3 2 1 F R
Fruit cake/sponge cake/sponge pudding - homemade	7 6 5 4 3 2 1 F R
Fruit tart/jam tart/doughnut/Danish pastry - shop bought	7 6 5 4 3 2 1 F R
Fruit tart/jam tart - homemade	7 6 5 4 3 2 1 F R
Milk pudding, e.g. rice/tapioca/macaroni	7 6 5 4 3 2 1 F R

What type of milk do you use for milk pudding?

1. Whole milk
2. Semi-skimmed milk
3. Skimmed milk
4. Canned milk pudding - ordinary
5. Canned milk pudding - low fat

## FRUIT

How often do you have fruit canned in syrup?	7 6 5 4 3 2 1 F R
How often do you have fruit canned in juice?	7 6 5 4 3 2 1 F R
How many apples do you have per week?	.....
How many pears do you have per week?	.....
How many oranges or grapefruit do you have per week?	.....
How many bananas do you have per week?	.....
How often do you have other fruit, e.g. peach/nectarine, grapes, kiwi, strawberries, melon?	7 6 5 4 3 2 1 F R

## EGGS & MILK PRODUCTS

How many eggs do you usually eat per week?	.....
What type of milk do you usually have?	
1. Whole milk	
2. Semi-skimmed milk	
3. Skimmed milk	

☐ 94

☐ 95

☐ 96

☐ 97

☐ 98

☐ 99

☐ 100

☐ 101

☐ 102

☐ 103

☐ 104

☐ 105

☐ ☐ 106-107

☐ ☐ 108-109

☐ ☐ 110-111

☐ ☐ 112-113

☐ 114

☐ ☐ 115-116

☐ 117

4. Soya milk
5. Goats milk
6. More than one type
7. None

How much milk do you drink per day in tea/coffee/milky drinks/cereals?

1. None
2. Half a pint or less
3. Between half a pint and one pint
4. One pint or more

How much cream do you use per week (1 tablespoon=20g; small carton=150g; large carton=300g)?

..... g

How much cheese (excluding cottage cheese) do you eat per week?

..... g

How often do you have cottage cheese?

7 6 5 4 3 2 1 F R

## FATS

What do you usually spread on bread?

1. Butter
2. Polyunsaturated margarine/spread
3. Other soft margarine/spread (tub) (NOT olive spread)
4. Hard margarine (block)
5. Low fat spread - polyunsaturated
6. Low fat spread - other
7. Very low fat spread (25% fat)
8. Olive oil spread
9. Bread eaten dry

How much butter/margarine/spread do you eat per week?

..... g

How often do you have food that is fried,

e.g. fish/onions/mushrooms/tomatoes/eggs?

7 6 5 4 3 2 1 F R

What type/brand of fat do you use for frying?

.....

What type/brand of fat do you use for chips?

.....

What type/brand of fat do you use for roast potatoes?

.....

What type/brand of fat do you use for homemade cake?

.....

What type/brand of fat do you use for homemade pastry?

.....

## DRINKS

How many cups of tea do you have per day?

.....

☐ 118

☐ ☐ ☐ 119-121

☐ ☐ ☐ 122-124

☐ 125

☐ ☐ 126-127

☐ ☐ ☐ 128-130

☐ 131

☐ 132

☐ 133

☐ 134

☐ 135

☐ 136

☐ ☐ 137-138

How many teaspoons of sugar/honey per cup?	.....	<input type="checkbox"/> 139
How many cups of coffee do you have per day?	.....	<input type="checkbox"/> <input type="checkbox"/> 140-141
How many teaspoons of sugar/honey per cup?	.....	<input type="checkbox"/> 142
How often do you have fruit juice?	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 143
How often do you have fruit squash (NOT low calorie)?	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 144
How often do you have fizzy drinks (NOT low calorie)?	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 145
How often do you have low calorie squash or fizzy drinks?	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 146
How often do you have a glass of water (tap /mineral) ?	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 147
In a typical week, how often do you have the following drinks and, when you have them, how many do you drink?		
Beer/lager/stout	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 148
Amount per occasion	..... Pints	<input type="checkbox"/> <input type="checkbox"/> 149-150
Cider	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 151
Amount per occasion	..... Half Pints	<input type="checkbox"/> <input type="checkbox"/> 152-153
Wine	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 154
Amount per occasion	..... Glasses	<input type="checkbox"/> <input type="checkbox"/> 155-156
Sherry/port/vermouth	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 157
Amount per occasion	..... Glasses	<input type="checkbox"/> <input type="checkbox"/> 158-159
Spirits/liqueurs	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 160
Amount per occasion	..... Measures	<input type="checkbox"/> <input type="checkbox"/> 161-162

### SOUPS & SAUCES

How often do you have the following soups & sauces?

Vegetable-based soups, e.g. vegetable/minestrone/carrot	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 163
'Cream of' soups, e.g. chicken, mushroom, tomato	7 6 5 4 3 2 1 F R	<input type="checkbox"/> 164

Sauces such as curry, sweet & sour, etc.	7 6 5 4 3 2 1 F R
Mayonnaise, e.g. in coleslaw/potato salad/sandwiches	7 6 5 4 3 2 1 F R
Salad cream	7 6 5 4 3 2 1 F R
Other dressings, e.g. French/thousand island/blue cheese	7 6 5 4 3 2 1 F R

☐ 165☐ 166☐ 167☐ 168**HEIGHT, WEIGHT & ACTIVITY**What is your height? ..... ft .....ins **OR** ..... cm☐☐☐ . ☐ 169-172What is your weight? ..... st .....lb **OR** ..... kg☐☐☐ . ☐ 173-176

How physically active is your occupation?

- 1 Not very active
- 2 Moderately active
- 3 Very active
- 4 Not working

☐ 177

How physically active is your leisure time?

- 1 Not very active
- 2 Moderately active
- 3 Very active

☐ 178

Questions for women only..

Are you pregnant?

Yes / No

☐ 179

Are you breast feeding?

Yes / No

☐ 180

## **1 e Study protocol**



**Are post-prandial hyperglycemia and post-prandial hypertriglyceridemia simultaneously harmful for both macro and microcirculation in diabetes?**

*A strong clinical implication.*

Post-prandial hyperglycemia has recently been suggested as an independent risk factor for cardiovascular disease (1-2). However, recent evidence suggests that postprandial hyperglycemia may also be a risk factor for micro-angiopathic complications, particularly retinopathy (3-5).

It is well recognized that an altered endothelial function plays a key role in the pathogenesis of cardiovascular disease and also in diabetes mellitus (6). Previous studies have already shown that retinal vasoconstriction and reduced retinal blood flow precede the onset of diabetic retinopathy (7) whilst diabetic retinopathy can further be either associated (8-9) or even predictive of future cardiovascular disease (18).

It has already been demonstrated that postprandial hyperglycemia and postprandial hypertriglyceridemia have an independent but cumulative effect in inducing an endothelial dysfunction (19). Of relevance, they are often simultaneously increased in the postprandial state in diabetic patients (1).

**Study Objectives**

Previous studies have already highlighted that endothelial dysfunction is involved in the pathogenesis of both cardiovascular disease and retinopathy, which are often

associated and inter linked (8,9 and 18). Postprandial hyperglycemia has been proposed as a common risk factor for these complications, the aim of this study is to evaluate, for the first time simultaneously, the response of endothelium to postprandial glucose. Furthermore, since postprandial hyperglycemia and postprandial hypertriglyceridemia have been shown to have an independent, but cumulative effect in worsening the endothelial function (19), this study, for the first time, will address the effect of glucose and fat load, alone or in combination, on endothelial function at the level of both arm and retina of diabetic patients.

## **Methods**

After informed consent is obtained, we will recruit 25 patients with Type 2 diabetes mellitus at various General Medical practices as well as acute hospitals across Coventry and Warwickshire in UK and 25 matched healthy subjects as control group. The control group of healthy patients will be selected from similar geographic area and matched for ethnicity.

This number of subjects to be recruited is based on the previous studies in this area (6-8). All subjects will be matched for age, sex, ethnicity, BMI, lipid profile and HbA1c. Only non smokers subjects and with a normal resting ECG, Blood pressure and no history of vascular disease will be selected. Patients on aspirin, lipid-lowering agents, or supplemental vitamins will be excluded as they can influence endothelial function. All subjects will be recommended to consume the habitual diet during the entire period of the study.

The subjects will be invited to UHCW Diabetes centre for Physiologic studies. This involves attending UHCW for 3 half days of study (5 hours each day). They will be overnight fasting for 10 hours and attend the centre in the morning. They will eat, in randomized order, on different days, 3 different menus: (1) a high-fat meal; (2) 75 g glucose alone; (3) a high-fat meal plus 75 g glucose. Blood samples will be drawn at 0, 1, 2, 3, and 4 hours, and the following variables will be assayed: plasma glucose, triglyceridemia, plasma insulin, flow-mediated vasodilatation (FMD) of the brachial artery, retinal blood flow (see below for the details), and skin capillary reactivity.

#### Endothelial Function

Endothelial function will be evaluated by measuring flow-mediated vasodilatation (FMD) of the brachial artery as previously described (19). Vasodilatation responses of the brachial arteries will be measured by ultrasound technique by a skillful examiner. The validity of this method has been previously confirmed (20). Briefly, the diameter of the brachial artery will be measured from B-mode ultrasound images with a 7.5-MHz linear array transducer (SSH-160A, Toshiba). Flow velocity in the brachial artery will be measured with a pulsed Doppler signal at a 70° angle to the vessel, with the range gate (1.5 mm) in the centre of the artery. The brachial artery will be scanned in the antecubital fossa in a longitudinal fashion. Depth and gain settings will be optimized at the beginning of the study



and will be kept constant throughout the recording period. When a satisfactory transducer position will be found, the surface of the skin will be marked, and the arm remained in the same position throughout the study. Each subject will lie quietly for 10 minutes before the first scan. At the end of each test, the subjects will lie quietly for 15 minutes. Then, sublingual nitroglycerin (0.3 mg) will be administered and 3 minutes later the last measurements will be performed.

Response to nitroglycerin will be used as a measure of endothelium-independent vasodilatation. All studies will be performed in a quiet and temperature controlled room (22°C to 23°C).

After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff placed around the forearm will be inflated with a pressure of 250 to 300 mm Hg and will be released after 5 minutes. The measurements of diameter and flow velocity will be continuously performed from cuff inflation to due time after cuff deflation. The ultrasound images will be recorded on a super VHS videocassette recorder (BR-S601 mol/L, Victor), and the arterial diameter will be measured at a fixed distance from an anatomical marker with ultrasonic calipers by two independent observers.

Measurements will be taken from the anterior to the posterior interface between the media and adventitia ("m" line) at the end of diastole, coincident with the R wave on a continuously recorded ECG (20). The diameters of 4 cardiac cycles will be analyzed for each scan, and the measurements will be averaged. Diameter measurements for the reactive hyperemia will be taken 45 to 90 seconds after cuff

deflation to measure peak diameter (20). Responses of the vessel diameters to the reactive hyperemia and nitroglycerin will be expressed as the percent increase above the baseline value of the diameter. Blood flow will be calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. The increase in the blood flow will be calculated by dividing the maximum flow within the first 15 seconds after the cuff deflation by the flow at baseline (20).

#### Ocular blood flow measurements:

Perfusion parameters in the superior temporal regions of the neuroretinal rim and peripapillary retina were measured for each step of the experiment using the HRF system. The HRF principle has previously been described in detail [10,11]. In short, this noninvasive technique combines laser Doppler flowmetry using an infrared laser diode with wavelength of 780 nm and a power of 200  $\mu$ W, with the confocal scanning laser principle. The HRF scanning system enables the measurement of back-scattered intensity for a retinal area of approximately 2.7 mm x 0.7 mm ( $10^\circ \times 2.5^\circ$  scan), mapped with a resolution of 256 points x 64 lines [10]. The total time for scanning and data acquisition is 2.048 sec. Each of the 64 horizontal lines is scanned 128 times with a line repetition rate of 4000 Hz. For each retinal point in the scanned area, the back-scattered intensity can be measured as a function of time to produce an intensity-time curve. The signal suffers a fast-



Fourier transformation, which results in a power spectrum for each retinal point.

This spectrum is used to calculate the variables volume, flow and velocity for each retinal point; the values are given as the mean and standard deviation of the measurements and are displayed in arbitrary units.

In this study, values of capillary volume, flow and velocity were recorded for all images using a 10 x 10 pixel measurement frame, avoiding areas with large vessels.

Three to five HRF measurements will be recorded by the same experienced investigator at the end of each step of the experiment; only the best quality images were selected for statistical purposes. Quality inclusion criteria were lack of movements during the recording and good illumination of the image. Images were included if the direct current (DC) values were between 70 and 200 [12,13].

Measurements were performed first on one location on the temporal NRR (TNRR) using a central alignment technique [14] and then on one location on the temporal retina (TR) while avoiding the large vessels.

The non invasive measurement takes 10-15 minutes.

#### Retinal vessels reactivity measurements:

Retinal vessel reactivity will be measured using the retinal vessel analyser (RVA, IMEDOS GmbH). The RVA measures the diameter of the column of the red blood cells. For measurements, the fundus camera is adjusted to the dilated pupil, and a

clear fundus image with good contrast and no reflections is obtained on the monitor. The RVA produces one vessel width measurement, expressed in units of measurement (UM), for each segment length of 12.5 UM. An optoelectronic shutter controlled by a special program running on the RVA computer, inserted in the camera, interrupts the observation light (530–600 nm, irradiance at the fundus  $\sim 1.96 \times 10^{-4} \text{ W/cm}^2$ ) over the entire  $30^\circ$  visual field of the retinal camera and produces a bright-to-dark contrast ratio of at least 25:1 (flicker provocation). After the baseline recording of 50 seconds, three flicker periods of 20 seconds each will be applied, interrupted with 80 seconds of still illumination.(15). The maximum vasomotion in response to flicker stimulation will be measured in two chosen segments of the major temporal inferior branch of the arteriole and venule. The point of maximum dilation will be determined for each flicker stimulation as the highest 1-second mean vessel diameter during the period of 50 seconds after the beginning of the corresponding flicker stimulus. Three points of maximum dilation will be obtained in all measuring sites (proximal and distal in arterioles and venules) and all will be expressed as a percentage of the corresponding first baseline value (16).

Peripheral vasospasm assessment by means of capillary microscopy.

All the subjects will undergo microscopic examination of their nail fold capillaries as described previously(17). The skin of the nail fold will be made transparent by a drop of oil. A light microscope computer will allow the observed blood flow to

7

recorded and analyzed. During nailfold-capillaroscopy, the nail fold area is cooled at -15 C for 60 seconds by rapidly decompressing carbon dioxide. The measurement takes 10-15 minutes and there will be no discomfort for the patient.

#### High-Fat Load

The standardized high-fat meal will consist of whipping cream and will contain 75 g of fat, 5 g of carbohydrates, and 6 g of protein per m<sup>2</sup> body surface area (19).

The corresponding caloric intake will be 700 kcal/m<sup>2</sup>.

#### Biochemical Measurements

Glycemia, triglyceridemia and insulinemia will be evaluated by standard methods.

#### **Statistical Analysis**

We suppose that each variable will have a normal distribution (6-8). However, the Kolmogorov-Smirnov algorithm will be used to determine whether each variable has a normal distribution. Comparisons of baseline data among the groups, according to the various polymorphisms, will be performed with the unpaired Student's *t* test or Wilcoxon test, according to the distribution. The changes in variables during the tests will be assessed by 2-way ANOVA with repeated measures. If difference reaches statistical significance, post hoc analyses with a 2-tailed paired *t* test will be used to assess differences at individual time periods in



the study, with Bonferroni correction used for multiple comparisons, or Wilcoxon's matched pair test. Statistical significance will be defined as  $p < 0.05$ .

### **Expected Results**

This study can show for the first time:

- if postprandial hyperglycemia is able to induce simultaneously an alteration of blood flow in both macro and microcirculation. In particular, in vessels involved in specific complications of diabetes, such as cardiovascular disease and retinopathy.
- If postprandial hypertriglyceridemia is able to induce simultaneously an alteration of blood flow in both macro and microcirculation. In particular, in vessels involved in specific complications of diabetes, such as cardiovascular disease and retinopathy.
- If the combination of postprandial hyperglycemia and postprandial hypertriglyceridemia is able to induce simultaneously an alteration of blood flow in both macro and microcirculation. In particular, in vessels involved in specific complications of diabetes, such as cardiovascular disease and retinopathy.

These results may constitute key evidence that controlling postprandial hyperglycemia can be helpful not only for preventing cardiovascular disease in diabetes, but also retinopathy.

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1 f Figure - Physiology lab, UHCW

Physiology laboratory picture shows a subject in a resting position with FMD scan of his right brachial artery being performed. The operator is looking at the ultrasound machine and the personal computer shows the VIA software tracking the arterial wall. The 12 MHz probe is held in position by a specially made stereotactic clamp and the automatic blood pressure machine can be seen below the table. It is connected to a cuff placed on the subjects' upper forearm.

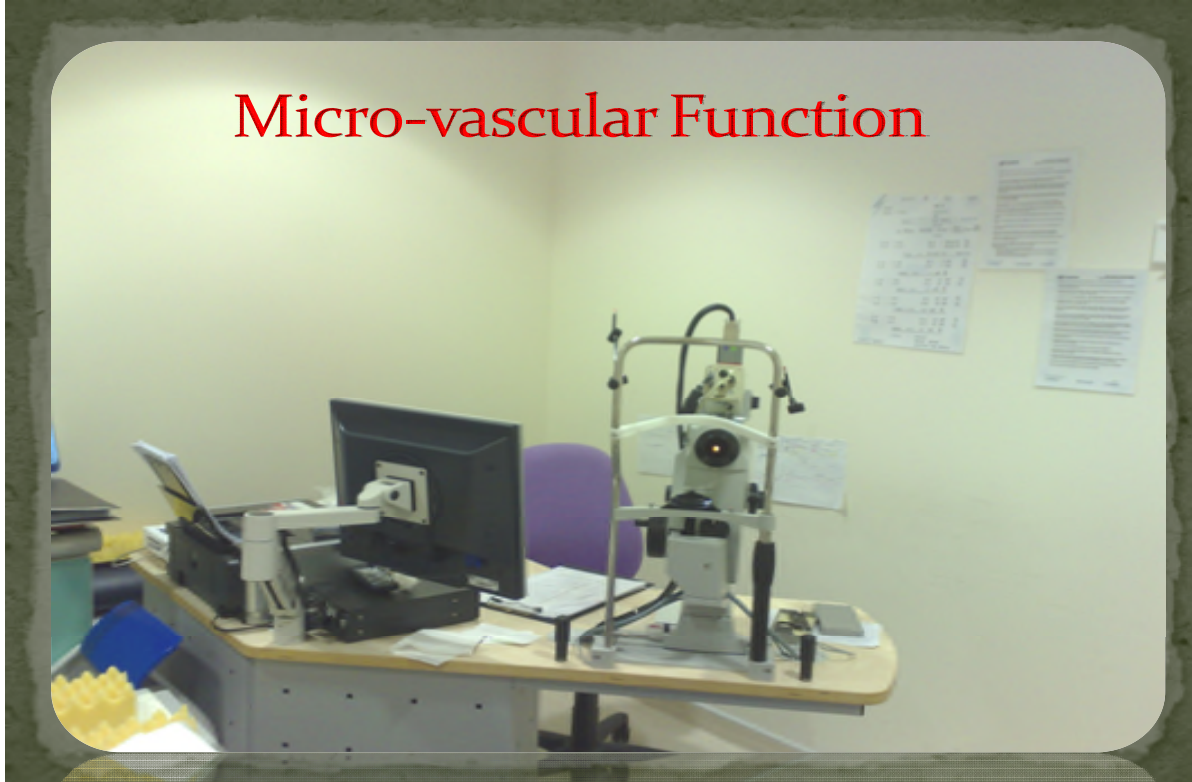


1 g Picture VIVID 7 ECHO machine

Standard ECHO machine which uses ultrasound technology to scan the brachial artery in 2D mode. The machine is connected to a 12 MHz probe which scans the brachial artery. The machine is connected to the personal computer using a frame grabber which transmits the image of the brachial artery to the VIA software which tracks the walls and measures the FMD values.



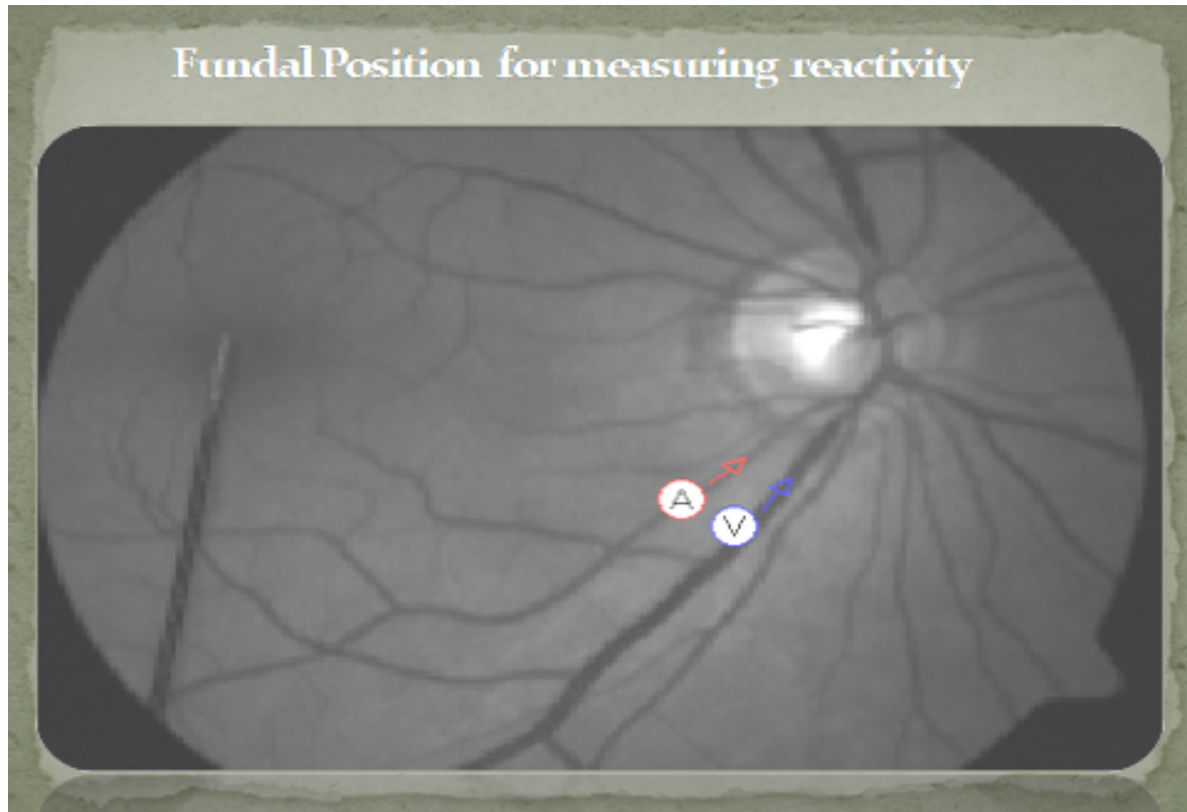
## Micro-vascular Function



1 h Figure RVA system with Fundus camera and CCD (Charge coupled device) device

The above picture shows the RVA system with the camera, CCD camera and the personal computer and printer installed on a single table. The computer has the software installed, which can track the retinal vessel walls for reactivity measurements. The subjects will sit in front of the camera with the right eye positioned looking directly into the camera and light. Once the subject is comfortable with the position, height and light, the measurements are started as per protocol.





# 1 i Figure

RVA picture to identify the best area for artery and vein scanning for the ROI

The above picture shows a still picture of the right eye/fundus with optic disc and the radiating retinal artery and vein. The metal pointer is at the centre of the macula. Both the artery and vein are marked in the inferior temporal region, at which point the reactivity measurements are undertaken. Repeat scans will pick the same spot to get the best results to check for dynamic shifts in reactivity measurements.

## **Appendix II**

### **Protocols and examples**

<b>MICRO VASCULAR STUDY</b> Flow mediated dilatation (FMD) and Retinal vessel Analysis (RVA) Scanning Schedule <span style="float: right;">Date:</span>			
8.00-8.30 AM	Check list Blood pressure Weight, BMI, BMR and Fat %		
Left arm Venflon and Bloods		Consent check 1 % tropicamide drops right eye Urine sample	
		Subject 1	Subject 2
8.35-9.00 AM	Baseline	FMD	RVA
9.05– 9.30 AM	Baseline	RVA	FMD
<u>75 Grams Glucose drink -----OGTT</u>			
10.25-10.45AM	1 Hour Post	FMD	RVA
10.45-11.05 AM	1 Hour Post	RVA	FMD
Blood test 1 Hour post			
11.25-11.45 AM	1 Hour Post	FMD	RVA
11.45-12.05 PM	1 Hour Post	RVA	FMD
Blood test 2 Hour post			
12.25-12.45 PM	1 Hour Post	FMD	RVA
12.45-13.05 PM	1 Hour Post	RVA	FMD
Blood test 3 Hour post			
13.25-13.45 PM	1 Hour Post	FMD	RVA
13.45-14.05 PM	1 Hour Post	RVA	FMD
Blood test 4 Hour post Test Finish- Venflon removed- Lunch			

2 a. Figure Protocol used for the study

The detailed protocol followed for the physiological tests performed each day of the study.

The figure clearly shows that the subjects usually attended the sessions before 8 AM in the

morning. There were usually 2 subjects who were studied each day. After confirming the identity and consent from each subject, the baselines of both macro- and micro-vascular function was performed on both subjects by 2 operators, respectively. Then baseline blood samples were obtained. Oral glucose (70 grams) was ingested by both subjects and 1 hr after that (5 minutes before the hr ), RVA was performed on 1 subject by 1 operator whilst the other had FMD performed by the other operator. Then they swapped over within 15 minutes. After 1 hr post blood samples, the subjects rested until the 2 hr scan. This procedure continued till they finished all 4 post scans and blood tests.

The final scan after the FMD was repeated after 250 µgrams of sublingual nitroglycerine (GTN) was administered. The protocol required that an FMD scan was performed at the end of 5 minutes after the GTN was administered. FMD was continuously monitored and recorded during this time. At the end of this scan the subject had the final 4 hr scan on the RVA with the GTN on board. The same protocol was followed for the other subject at the 4 hr point.

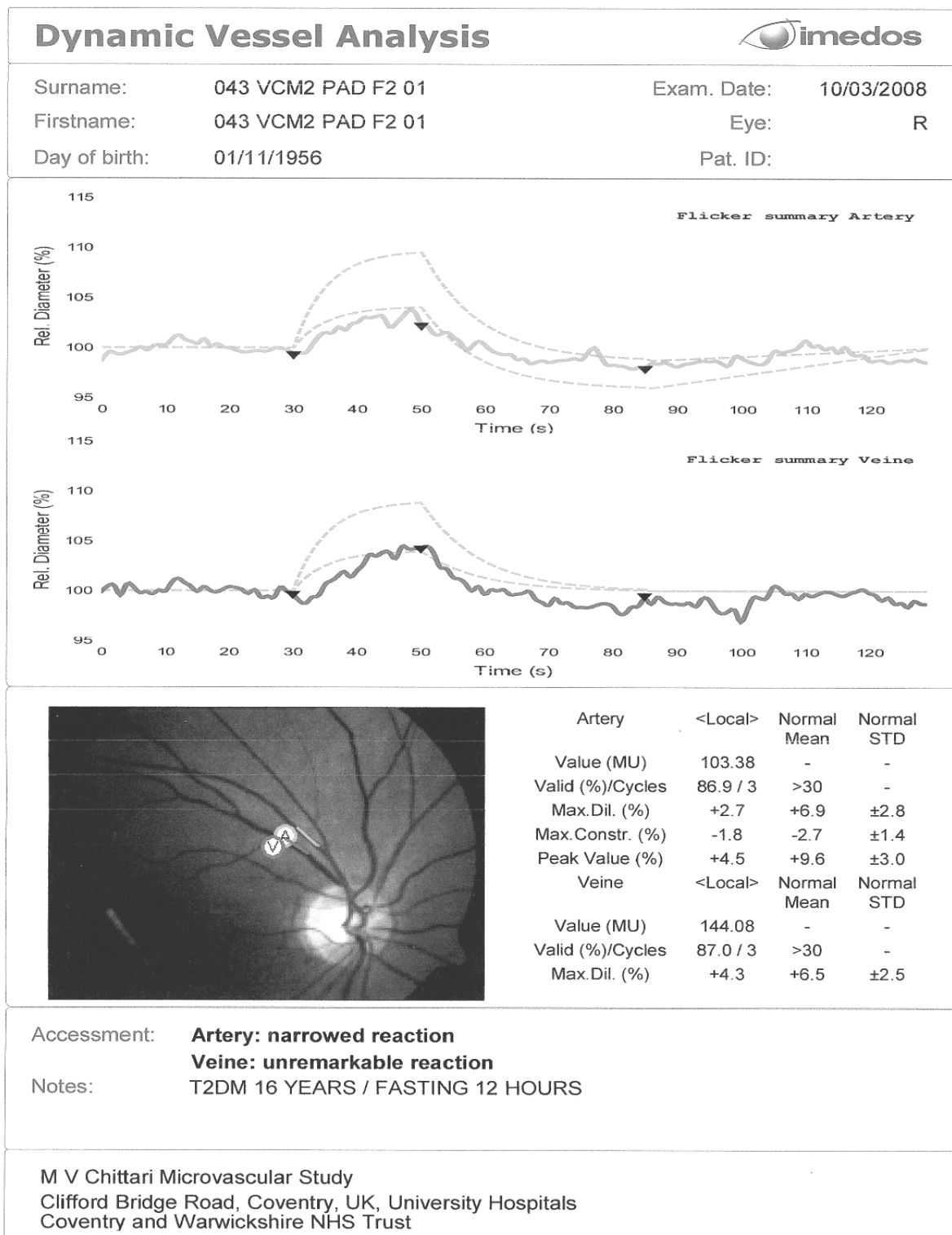
084 VCM2 SAN G101	
Date 28/7/08	Day 1
Session 1 PRE fasting	Tape Number: 10
DOB: 7/10/76	Tape start: 0:42:13
VIA Session: (Final) 3	Tape end: 0:44:16
Diabetic yes 1 No (2)	Room Temp. 21°C
<b>FMD (Flow Mediated Dilatation) Brachial Art:</b>	
Baseline Blood Pressure 134/78	Baseline Heart Rate: 54-55
Baseline Diameter VIA (mm): 10.2-10.5	Baseline PW doppler 84.55
Baseline Diameter ECHO (cm): 0.50	Blood Flow calculated:
<b>Compression 300 mmHg</b>	
Diameter VIA (mm): 10.2-10.5	<b>FMD / GTN Study</b>
Diameter ECHO (cm): 0.49	Baseline Blood Pressure:
Heart Rate 51-52	Baseline Heart Rate:
PW doppler: 62.73	Baseline Diameter VIA (mm):
	Baseline Diameter ECHO (cm):
<b>Post Cuff release</b>	<b>250 mcg. GTN sublingual</b>
PW doppler 180.01	Peak Diameter (5 mts) VIA (mm):
Post cuff HR 56-57	Peak Diameter (5 mts) ECHO (cm):
Diameter (60sec) Peak VIA (mm): 10.4-10.7	Peak HR (5 mts)
Diameter (60sec) Peak ECHO (cm): 0.50	Blood Pressure:
Final Blood Pressure 126/77	10.4-10.7
Blood Flow (peak) calculated:	

Clinical Sciences Research Institute  
 Clinical Sciences Building  
 University Hospital - Walsgrave Campus  
 Clifford Bridge Road  
 Coventry CV2 2DX United Kingdom  
 Tel: +44 (0)24 7696 8652  
 Fax: +44 (0)24 7696 8653  
 www.warwick.ac.uk/go/clinsci



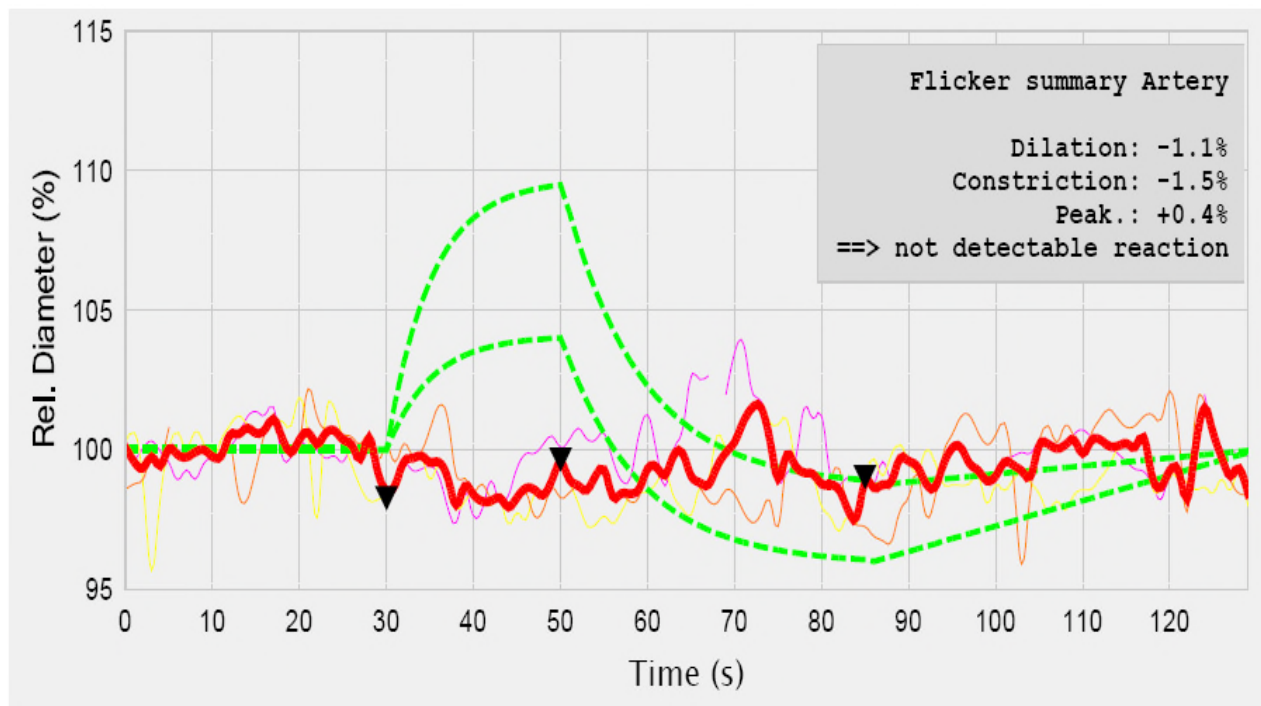
### 2 b Figure

Real time standard record sheet. VIA and ECHO parameters recorded live as the scan is done. Record of FMD scanning for each subject, which included recording all cardiovascular parameters obtained from both standard ECHO machines with 2D scan parameters, as well as VIA software parameters. These were recorded at all 3 stages of scanning, including at baseline, cuff inflation and after deflation.



2 c Figure

Final analysis of both arterial and venous reactivity in the retina: Real time report of RVA scans on a subject with both arterial and venous reactivity measurements, along with location and validity measurements clearly shown. Two graphs, one arterial and the other venous reactivity measurements to flicker light, are shown with dilatation % recorded. Validity measurements for each scan were generated by the software.



2 d Figure

The above example from the study clearly shows a difference in the arterial reactivity measurements between the top scan results versus the bottom. They happen to show the scan results between a diabetic subject, on the top graph, compared with a non-diabetic (ND) subject in the bottom graph. The reactivity in the diabetic subject appears to be low in the fasting state compared with the control subject.

## Static Vessel Analysis

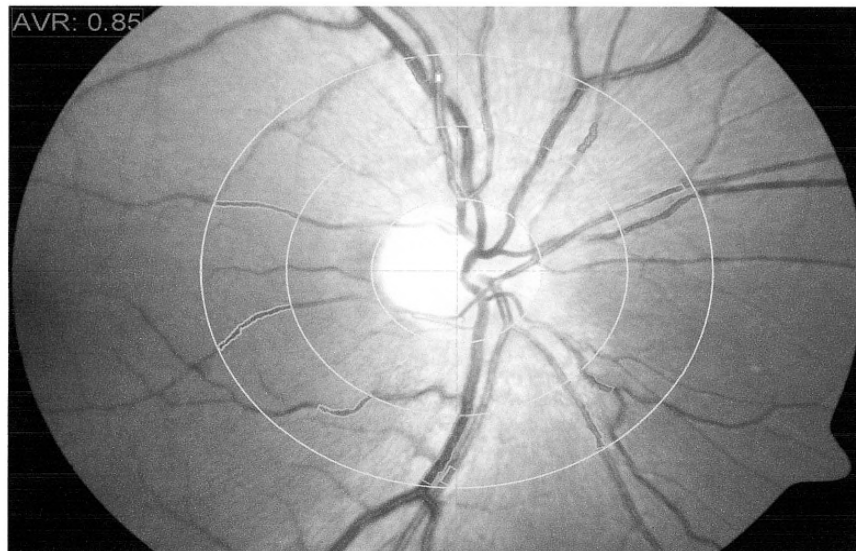
### Patient

Name: 084 VCM2 SAN F2 01  
 First Name: 084 VCM2 SAN F2 01  
 Date of Birth: 10/07/1976  
 Pat.-ID:  
 Date: 07/08/2008



Address: M Varma Chittari Microvascular Study  
 Coventry, UK, University Hospitals  
 Coventry and Warwickshire NHS Trust

Coventry, UK

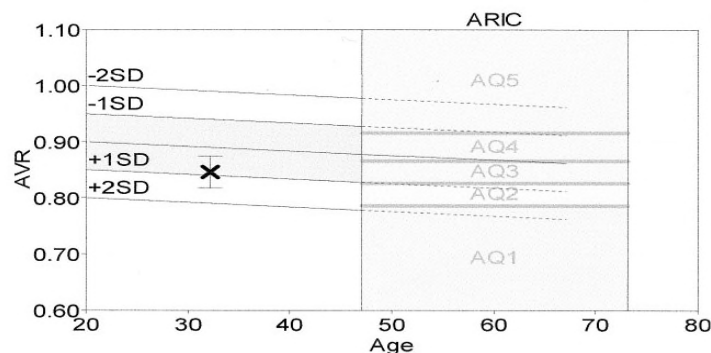


Eye: R  
 Image ID: 04

AV-Ratio (AVR): 0.85

arterial vessels: 9  
 Artery Equivalent (CRAE): 165.5

venous vessels: 5  
 Vein Equivalent (CRVE): 195.6



The diagram is a statistical appraisal helper for the comparison of the individual examination result with the reference data of the ARIC study "amongst others" Wong TY et al: Retinal microvascular abnormalities and incident stroke. The Atherosclerosis Risk in Communities Study. Lancet 2001, 258:1134-1140" and on the Munich normal data study 2006 respectively. The diagnosis stays in the accountability of the doctor.

Comment:  
 NON DM/ FASTING 12 HOURS / DAY 2 PRE

2 e Figure Final analysis of Arterio-venous ratio.

The above figure shows the still picture of the right eye/fundus, with arterial and venous markings in a circular panel created by the DVA software. The system generates the report once the arteries and veins within the area are highlighted. The final ratio of the arterial versus venous size on this picture is 0.85. The bottom part of the picture is a graph with the obtained value marked as a cross. The graph is based on ARIC study findings for AV ratio.

ARIC- Atherosclerosis risk in communities study.



VCM2 – STRESS STUDY – BOX 1											
		1	2	3	4	5	6	7	8	9	
Pre (G)	A	G101P1	G101P2	G101S1	G101S2	G101 URINE	F205P1	F205P2	F205S1	F205S2	4 hr post
1 hr post	B	G102P1	G102P2	G102S1	G102S2	G102S3	C301P1	C301P2	C301S1	C301S2	1 hr post
2 hr post	C	G103P1	G103P2	G103S1	G103S2	C302P1	C302P2	C302S1	C302S2	C302S3	2 hr post
3 hr post	D	G104P1	G104P2	G104S1	G104S2	C303 URINE	C303P1	C303P2	C303S1	C303S2	3 hr post
4 hr post	E	G105P1	G105P2	G105S1	G105S2		C304P1	C304P2	C304S1	C304S2	4 hr post
Pre (F)	F	F201P1	F201P2	F201S1	F201S2	F201 URINE	C305P1	C305P2	C305S1	C305S2	4 hr post
1 hr post	G	F202P1	F202P2	F202S1	F202S2	F202S3					1 hr post
2 hr post	H	F203P1	F203P2	F203S1	F203S2						2 hr post
3 hr post	I	F204P1	F204P2	F204S1	F204S2						3 hr post

- 80° C FREEZER - Bottom Shelf -

## 2 f Figure

Sample storage database for the study at the freezers.

The above figure is an example of anonymised coding used in the study for all samples stored at -80°c.

# VCM2 microvascular study

## Day 1 (Glucose load)

Research code...084 SAN.....

Date: 28/7/08

7/10/76

1/2 fruit watermelon 12 hours before Non-DM

	RV artery (V)	FMD (N)	RV vein (V)	A/V ratio (No.)	Glucose	LDL	TG.
Pre-	+2.3 (86%)	1.34 (28)	+1.9 (87%)	0.82 (9/5)	4.8	4.6	1.50
1 hr.	+1.8 (83%)	1.78 (44)	+1.5 (65%)	0.87 (9/5)	8.1	—	1.57
2 hr.	+3.2 (83%)	1.52 (41)	+2.1 (85%)	0.89 (9/5)	4.6	—	1.49
3 hr.	+2.0 (87%)	-0.07 (49)	+1.5 (86%)	X	3.7	—	1.52
4 hr.	X	2.17 (35)	X	X	4.3	—	1.42
4hr.+GTN	+3.0 (77%)	12.36 (35)	+2.6 (85%)	0.81 (9/5)	X	X	X

TC HDL  
6.5 1.2

## 2 g Figure

### Summary of individual scan results along with laboratory markers

The above chart shows a summary sheet with results from both micro- and macro-vascular reactivity noted. It also summarises time points for these results, along with plasma and serum markers. It also monitors the AV ratio for the time points.

## **Appendix III**

### **Statistical calculations**

One Sample		Kolmogorov-Smirnov Test T2DM OGTT				
T2DM	OGTT	Fasting	1 hr. post	2 hrs. post	3 hrs. post	4 hrs. post
N		21	21	21	21	21
Normal Parameters <sup>a</sup>	Mean	7.852	15.567	15.805	11.943	8.300
	Std. Deviation	2.0607	4.8950	3.5169	4.8667	4.2733
Most Extreme Differences	Absolute	.093	.110	.116	.129	.136
	Positive	.093	.110	.116	.129	.136
	Negative	-.069	-.062	-.065	-.083	-.107
Kolmogorov-Smirnov Z		.426	.504	.530	.592	.625
Asymp. Sig. (2-tailed)		.994	.961	.942	.874	.829

Table. 2.5.1 Kolmogorov test on mean serum glucose levels on OGTT for T2DM subjects. a. Test distribution is Normal. b. Calculated from data. It demonstrates that the values are normally distributed.

Descriptive Statistics    One-Sample Kolmogorov-Smirnov Test

T2 DM    FMD on OGTT	N	Mean	Std. Deviation	Minimum	Maximum
Fasting levels / pre-	20	2.5455	2.13417	-.93	7.81
1 hr post OGTT	20	1.5670	2.25323	-1.16	8.75
2 hr post	21	1.8300	3.07925	-.81	13.48
3 hr post	20	2.1060	2.69237	-1.76	9.90
4 hr post	21	2.5124	2.61959	-1.04	8.54
4 hour post and GTN (250 microgram)	20	34.45	9.848	19	57

Table 2.5.2 Kolmogorov test on mean FMD brachial artery levels on OGTT

for T2DM subjects. It demonstrates the descriptive values to calculate the test.

Descriptive Statistics Validity parameters -T2 DM FMD % on OGTT

T2 DM – FMD Noise levels % / validity	N	Minimum	Maximum	Mean	Std. Deviation
Fasting	20	16	55	35.94	10.086
1 hr post OGTT	20	21	65	37.00	12.990
2 hr post	21	13	63	35.19	13.167
3 hr post	20	16	56	38.30	11.145
4 hr post	21	21	60	34.56	10.002
4 hour post and GTN (250 microgram)	20	19	57	34.45	9.848

Table 2.6.1 Noise levels representing validity measurements for FMD % during OGTT on T2DM subjects.

The above table demonstrates that the validity measurements were within the parameters set by the software manufacturer. (The mean value at each point < 40 %)

Descriptive Statistics    Validity parameters - ND    FMD %    OGTT

ND – FMD Noise levels % / validity	N	Minimum	Maximum	Mean	Std. Deviation
Fasting	9	18	46	31.78	9.431
1 hr post OGTT	10	13	53	33.60	12.607
2 hr post	9	12	49	37.56	11.555
3 hr post	10	22	42	31.90	7.400
4 hr post	10	11	56	32.64	14.495
4 hour post and GTN (250 microgram)	10	11	57	33.91	15.005

Table 2.6.2 Noise levels representing validity measurements for FMD % during OGTT on ND subjects.

The above table demonstrates that the validity measurements were within the parameters set by the software manufacturer. (The mean value at each point < 40 %).

Descriptive Statistics Validity parameters- T2 DM RVR% on OGTT

T2DM – RVR % on OGTT Validity parameters		Descriptive Statistics			
T2DM – RVR Validity % on OGTT	N	Minimum	Maximum	Mean	Std. Deviation
RVA pre-	16	62	87	78.63	8.445
RVA 1 hr	20	64	91	78.05	7.156
RVA 2 hr	21	63	89	79.19	6.824
RVA 3 hr	21	42	90	76.00	11.975
RVA 4hr + GTN (250 microgram)	21	60	89	77.33	9.329

Table 2.6.3 Validity measurements for RVR % during OGTT on T2DM subjects.

The above table demonstrates that the validity measurements were within the parameters set by the software manufacturer. (The mean value at each point > 40 %)



Descriptive Statistics -Validity parameters - ND – RVA % on OGTT

ND – RVA Validity % on OGTT	N	Minimum	Maximum	Mean	Std. Deviation
RVA pre-	9	75	90	82.89	4.859
RVA 1 hr	10	44	87	77.80	12.621
RVA 2 hr	10	55	86	77.10	10.482
RVA 3 hr	10	29	87	70.40	17.232
RVA 4hr + GTN (250 microgram)	10	59	82	73.70	8.367

Table 2.6.4 Validity measurements for RVA % during OGTT on ND subjects.

The above table demonstrates that the validity measurements were within the parameters set by the software manufacturer. (The mean value at each point > 40 %)

T2 DM    FMD           Paired Samples           Correlations

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	Fasting & 1 hr. post glucose load	19	.513	.025
Pair 2	Fasting & 2 hrs. post glucose load	20	.622	.003
Pair 3	Fasting & 3 hrs. post glucose load	19	.482	.037
Pair 4	Fasting & 4 hrs. post glucose load	20	.622	.003

Table 6.2.7.1 Mean FMD on OGTT for T2DM subjects. Paired t test

It demonstrates that all the values are correlated.

T2 DM OGTT Paired Samples Test for FMD brachial

FMD % Values T2DM		Paired Differences					t	df	Sig. (2-tailed)
OGTT test					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	Fasting - FMD 1 hr post	1.08105	2.20077	.50489	.02032	2.14179	2.141	18	.046
Pair 2	Fasting - FMD 2 hr post	.65700	2.46942	.55218	-.49872	1.81272	1.190	19	.249
Pair 3	Fasting - FMD 3 hr post	.52105	2.55454	.58605	-.71020	1.75230	.889	18	.386
Pair 4	Fasting - FMD 4 hr post	.06950	2.15174	.48114	-.93755	1.07655	.144	19	.887
Pair 5	Fasting - FMD GTN (250 microgram)	-9.69789	4.15526	.95328	-11.70066	-7.69512	-10.173	18	.000

Table 6.2.7.2 Mean FMD values from baseline to 1 hour post OGTT appear to be statistically significant ( $P < 0.05$ ). Other time points and values displayed.

T2DM – RVA % on OGTT

Wilcoxon Signed Ranks Test - Test Statistics

T2DM RVA %	1 hr – pre-	2 hr – pre-	3 hr – pre-	4 hr – pre-with 250 GTN
Z	-1.933 <sup>a</sup>	-1.656 <sup>a</sup>	-1.683 <sup>a</sup>	-1.907 <sup>a</sup>
Asymp. Sig. (2-tailed)	.053	.098	.092	.057
a. Based on positive ranks.				
b. Based on negative ranks c. Wilcoxon Signed Ranks Test				

Table 6.2.7.3 P value for RVA levels on OGTT in T2DM subjects.

The table demonstrates that, on Wilcoxon signed ranks test, mean RVA values from baseline to 1 hour post OGTT appear to be statistically significant ( $P=0.05$ ).

Other time points and values displayed.

ND subjects		OGTT										One-Sample Kolmogorov-Smirnov Test	
ND subjects OGTT - FMD% RVA%		FBS	FMD pre-	FMD 1hr	FMD 2 hr	FMD 3 hr	FMD 4 hr	RVA pre-	RVA 1 hr	RVA 2 hr	RVA 3 hr	RVA 4hr + GTN (250 microgram)	
N		10	9	10	9	10	10	9	10	10	10	10	
Normal Parameters <sup>a,b</sup>	Mean	5.230	3.7100	1.6920	.8122	2.5490	2.1210	3.222	3.300	2.770	2.640	3.370	
	Std. Deviation	.6219	3.5303 9	2.68517	2.80229	1.87830	3.39750	1.7998	1.7913	.8994	1.0752	1.7957	
Most Extreme Differences	Absolute	.192	.204	.149	.247	.210	.126	.151	.212	.184	.133	.151	
	Positive	.192	.204	.149	.247	.210	.126	.140	.110	.183	.133	.129	
	Negative	-.119	-.181	-.091	-.195	-.116	-.120	-.151	-.212	-.184	-.122	-.151	
Kolmogorov-Smirnov Z		.608	.613	.472	.741	.665	.400	.453	.669	.581	.422	.477	
Asymp. Sig. (2-tailed)		.853	.846	.979	.642	.768	.997	.986	.762	.889	.994	.977	

Table 6.2.7.4 ND OGTT, FMD and RVA data for normal distribution test.

a. Test distribution is Normal b. Calculated from data.

Test to demonstrate that the samples in the ND group were normally distributed

ND OGTT Paired Samples t Test

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	Fasting & 1 hr. post glucose load	-1.6100	2.1010	.6644	-3.1130	-.1070	-2.423	9	.038
Pair 2	Fasting & 2 hrs. post glucose load	-.7700	2.2126	.6997	-2.3528	.8128	-1.100	9	.300
Pair 3	Fasting & 3 hrs. post glucose load	.9600	.9407	.2975	-.2871	1.6329	3.227	9	.010
Pair 4	Fasting & 4 hrs. post glucose load	1.1900	.3542	.1120	-.9366	1.4434	10.625	9	.000

Table 6.2.7.5. Mean serum glucose levels on OGTT for ND subjects. Paired t test- Mean Serum Glucose levels on OGTT in ND subjects.

It demonstrates that, as compared with pre-prandial levels, serum glucose levels were increased up to 1 hour after the glucose stress showing statistical significance at 1, 3 and 4 hour post values versus baseline. ( $P < 0.05$ ).

ND-FMD % on OGTT Wilcoxon Signed Ranks Test - Test Statistics

ND - FMD %	1 hr - pre-	2 hr - pre-	3 hr - pre-	4 hr - pre-
Z	-1.007 <sup>a</sup>	-2.521 <sup>a</sup>	-.700 <sup>a</sup>	-1.244 <sup>a</sup>
Asymp. Sig. (2-tailed)	.314	.012	.484	.214

Table 6.2.7.6 P value for FMD levels on OGTT in ND subjects

Based on positive ranks.

Based on negative ranks

Wilcoxon Signed Ranks Test

The table demonstrates that, on Wilcoxon signed ranks test, mean FMD values from baseline to 2 hour post OGTT appears to be statistically significant ( $P < 0.05$ ). Other time points and values displayed.

GTN response	N	Arterial reactivity% ± SE	Standard deviation	P value T test for means
T2DM FMD	21	12.35 ± 0.94	4.30	0.29
ND FMD	10	14.69 ± 2.51	7.96	
T2DM RVA	22	1.76 ± 0.35	1.67	0.02
ND RVA	10	3.37 ± 0.56	1.79	

#### 6.2.7.7 FMD and RVA % after 250 micrograms sublingual nitroglycerine (GTN) administration

The above table reveals the arterial reactivity measurements to sublingual nitroglycerine at 250 micrograms. Both macro and micro vascular response in T2DM and ND are shown.

It appears that the macro vascular response to GTN as evidenced by FMD % in T2DM and ND are not significantly different ( $P = 0.29$ ) on independent samples t test.

It appears that micro vascular response to GTN as evidenced by RVA % in T2DM and ND appear to be significantly different ( $P = 0.02$ ) on independent samples t test.



T2 DM OGTT Paired Samples t Test									
		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. deviation	Std. Error Mean	Lower	Upper			
Pair 1	Fasting & 1 hr. post glucose load	-7.7143	4.0494	.8836	-9.5575	-5.8710	-8.730	20	.000
Pair 2	Fasting & 2 hrs. post glucose load	-7.9524	2.6206	.5719	-9.1453	-6.7595	-13.906	20	.000
Pair 3	Fasting & 3 hrs. post glucose load	-4.0905	3.7719	.8231	-5.8074	-2.3735	-4.970	20	.000
Pair 4	Fasting & 4 hrs. post glucose load	-4.4476	3.2432	.7077	-1.9239	1.0287	-.632	20	.534

Table 6.2.7.8 Mean serum glucose levels on OGTT for T2DM subjects.

Paired t test- Mean Serum Glucose levels on OGTT in T2DM subjects

It demonstrates that, as compared with pre-prandial levels, serum glucose levels were increased up to 2 hours after the glucose stress showing statistical significance at 1, 2 and 3 hour post values versus baseline. ( $P < 0.001$ ).

## **PUBLICATIONS**

RESEARCH ARTICLE

Open Access



# Metabolic endotoxaemia in childhood obesity

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## Abstract

**Background:** Childhood obesity is associated with chronic low-grade inflammation considered as a precursor to metabolic disease; however, the underlying mechanisms for this remain unclear. Studies in adults have implicated gut derived gram-negative bacterial fragments known as lipopolysaccharide or endotoxin, activating the inflammatory response, whilst the importance in childhood obesity is unclear. The aim of this research is to understand the relationship between circulating endotoxin in childhood obesity, and its association with inflammatory and cardiovascular (CV) injury biomarkers.

**Methods:** Fasted blood was obtained from children with varying degrees of obesity (age:  $13.9 \pm 2.3$ yr; BMI:  $35.1 \pm 5.2$  Kg/m<sup>2</sup>;  $n = 60$ ). Multiplex CVD biomarker immunoassays were used to determine systemic levels of inflammatory and vascular injury biomarkers, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin (IL-) 1 $\beta$ , 6, 8 and 10, plasminogen activator inhibitor-1 (PAI-1), soluble intercellular adhesion molecule type-1 (sICAM-1), matrix metalloproteinase-9 (MMP-9), myeloperoxidase (MPO) and vascular endothelial growth factor (VEGF) as well as endotoxin levels.

**Results:** Endotoxin levels demonstrated a significant and positive correlation with the markers for inflammation, vascular injury and atherogenesis (TNF- $\alpha$ :  $r^2 = 0.077$ ,  $p < 0.05$ ; PAI-1:  $r^2 = 0.215$ ,  $p < 0.01$ ; sICAM-1:  $r^2 = 0.159$ ,  $p < 0.01$ ; MMP-9:  $r^2 = 0.159$ ,  $p < 0.01$ ; MPO:  $r^2 = 0.07$ ,  $p < 0.05$ ; VEGF:  $r^2 = 0.161$ ,  $p < 0.01$ ). Males demonstrated significantly higher circulating endotoxin than females (Males:  $9.63 \pm 5.34$  EU/ml;  $p = 0.004$ ; Females:  $5.56 \pm 4.06$  EU/ml;  $n = 60$ ) in these BMI and age-matched cohorts.

**Conclusion:** The present study demonstrates for the first time a significant association between circulating endotoxin and biomarkers of metabolic risk in children as young as 11 years. Thus, endotoxin-mediated sub-clinical inflammation during childhood obesity may be a key contributor to T2DM and CVD development later in life.

**Keywords:** Endotoxin, Childhood obesity, Cardiovascular injury markers, Insulin resistance

## Background

Environmental, physical and nutritional factors appear critical in determining lifetime disease risk profile for cardiovascular disease (CVD), which is a leading cause of mortality worldwide [1–3]. CVD development in later life shows an increased risk when the condition is preceded by prior chronic inflammatory conditions [4–9]. Therefore the clinical value of determining the factors that induce an inflammatory response in early life

appears important to address. Clearly childhood obesity per se has a significant impact on disease risk, inflammation and CVD, however, adipose tissue (AT) may be an early contributor to metabolic dysfunction through prior systemic insults, which initiate an inflammatory response [7, 8, 10–14].

One cellular mechanism for an increased inflammatory response may arise through activation of the innate immune system as observed in human AT [14, 15]. Previous studies have shown that increased activation of the innate immune pathway may arise through excess circulating gut derived bacteria, known as lipopolysaccharide

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(LPS) or endotoxin; which represents fragments from the outer cell wall membrane of gram-negative bacteria [14–16]. In human AT, it appears endotoxin has an immediate impact on the innate immune pathway, acting via key receptors known as the toll like receptors (TLRs), which recognise antigens such as the LPS component, to initiate an acute phase response to infection [8, 14]. Stimulation of the TLRs leads to intracellular activation of NF $\kappa$ B, a key transcription factor in the inflammatory cascade that regulates the transcription of numerous pro-inflammatory adipocytokines [14, 15]. Therefore whilst *in vitro*, endotoxin may act as a mediator of inflammation through activation of NF $\kappa$ B, leading to a rapid response, in an *in vivo* situation that may be further exacerbated by an increasing fat mass, such as in obesity [17–21].

Clinical studies have also implicated gut derived endotoxin as a direct 'primary mediator' to activate the inflammatory state, contributing to metabolic disease, with current cross sectional data showing elevated systemic endotoxin levels in conditions of obesity, coronary artery disease, type 2 diabetes mellitus and fatty liver disease [8, 14–16], which is reduced with weight change [17, 21]. Studies in adults has also shown circulating endotoxin to be positively associated with waist, waist-hip-ratio, insulin levels, inflammatory cytokines as well as lipids, including total cholesterol, triglycerides, LDL-cholesterol and negatively associated with HDL-cholesterol [8, 17–21]. Recent studies in obese children and adolescents has demonstrated that systemic inflammatory cytokines such as plasminogen activator inhibitor-1 (PAI-1) and C-reactive protein (CRP) are elevated, along with vascular injury and atherogenesis markers, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule type-1 (ICAM-1) [21–24]. Whether such pathogenic biomarkers directly correlate with systemic endotoxin concentrations in obese children and adolescents is undetermined. We have examined and concluded that bacterial endotoxin is a potential biomarker of sub-clinical inflammation and early CVD risk in childhood obesity.

## Methods

### Subjects

A total of 60 (unless stated otherwise in the figure legend) obese children and adolescents with varying degrees of obesity (BMI: mean  $\pm$  (SD)  $35.1 \pm 5.2$  kg/m<sup>2</sup>; age:  $13.9 \pm 2.3$  years) were recruited among those referred for weight loss intervention to the obesity centre of the Istituto Auxologico Italiano. All subjects were above the age and sex adjusted 97th BMI percentile, which defines obesity according to the Italian BMI charts [25] and had an age range of 8–18 years. The Ethics Committee of the Italian Institute approved this study and informed

consent was obtained from all subjects and their parents. All subjects underwent an oral glucose tolerance test (1.75 g/Kg, up to a maximum of 75 g glucose in 250 ml of water) following an overnight fast. Plasma samples were drawn at baseline, after 30 min and 120 min, for determination of plasma glucose and insulin concentration. Categorisation of glucose tolerance status was made using the World Health Organisation criteria [20]. The impaired fasting glucose was defined by fasting glucose levels  $\geq 5.6$  mmol/l [26]. Blood samples were drawn for measurement of endotoxin, adiponectin and markers of inflammation and CVD. Blood pressure measurements were taken as previously described [11]. Insulin resistance was measured by HOMA-IR (fasting insulin  $\times$  fasting glucose/22.5) [27].

### Biochemical measurements

Serum endotoxin was assayed using a Chromogenic Limulus Amebocyte Lysate (LAL) test, which is a quantitative test for gram-negative bacterial endotoxin (Cambrex, New Jersey, USA) endotoxin-free vials were utilised throughout. Gram-negative bacterial endotoxin catalyzes the activation of a pro-enzyme in the Limulus Amebocyte Lysate (LAL). The initial rate of activation is directly determined by the concentration of endotoxin. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405–410 nm following termination of the reaction. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1–1.0 EU/mL range. Intra-assay CV  $3.9 \pm 0.46$ , inter-assay CV  $9.6 \pm 0.75$ . For the purposes of these studies all samples were run in duplicate within the same plate, therefore no inter-assay variability was observed in this study. To assess recovery of endotoxin within the assay, previous studies have utilised known concentrations of recombinant endotoxin (0.25 and 1.00 EU/mL) were added to diluted, pooled plasma to determine whether the expected concentration correlated closely with the actual observed value and whether there were any variations due to reaction with plasma contents [14]. Lyophilized endotoxin (*E. coli* origin) was used to generate a standard curve with the Chromogenic LAL test kit from Cambrex and produced a corresponding curve in accordance with the manufacturer's instructions. In plasma, the recovery of spiked endotoxin was  $82.0 \pm 3.3$  % efficient, similar recovery data were noted for serum. Plate to plate variability within the same experiment was  $7.4 \pm 0.9$  %, these findings were similar to those observed from assessment by Cambrex [14].

A multiplexed CVD Panel 1 immunoassay (Linco Research, Missouri, USA) was utilised to examine the circulating concentrations of the following inflammatory and CVD risk biomarkers: TNF- $\alpha$ , PAI-1 (tPAI-1, total)



**Table 1** Clinical and biochemical characteristics for obese, BMI and age-matched male ( $n = 24$ ) and female ( $n = 36$ ) subjects

Clinical and biochemical characteristics	Male Subjects ( $\pm$ SD)	Female Subjects ( $\pm$ SD)	<i>p</i> value
Age (yr)	14 $\pm$ 3	14 $\pm$ 2	-
BMI ( $\text{kg}/\text{m}^2$ )	34.0 $\pm$ 5.1	35.6 $\pm$ 5.3	-
Fasting Glucose (mmol/l)	4.4 $\pm$ 0.3	4.5 $\pm$ 0.5	N/S
2 h glucose, mmol/l	6.0 $\pm$ 1.0	5.7 $\pm$ 1.0	N/S
HOMA-IR	2.6 $\pm$ 1.2	3.3 $\pm$ 2.1	N/S
Endotoxin (EU/ml)	10.1 $\pm$ 5.4	53 $\pm$ 3.7	$p < 0.01^{**}$
CRP ( $\mu\text{g}/\text{ml}$ )	7.5 $\pm$ 8.3	90 $\pm$ 7.5	N/S
TNF- $\alpha$ (pg/ml)	5.0 $\pm$ 2.5	59 $\pm$ 5.8	N/S
PAI-1 (ng/ml)	27.0 $\pm$ 10.7	260 $\pm$ 15.7	N/S
sICAM-1 (ng/ml)	110.4 $\pm$ 37.4	1031 $\pm$ 47.9	N/S
Systolic BP (mmHg)	121.0 $\pm$ 10.8	1191 $\pm$ 8.5	N/S
Diastolic BP (mmHg)	74.0 $\pm$ 11.2	72.7 $\pm$ 8.5	N/S

Data are expressed as mean  $\pm$  SD. Significant differences in data between male and female subjects are highlighted ( $P$ -value  $^{**}$ ,  $p < 0.001$ )

CRP, soluble intercellular adhesion molecule type-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), MMP-9, MPO, VEGF and soluble endothelial selectin (sE-Selectin). The CVD Panel 1 immunoassay had a sensitivity of 16–50,000 pg/ml for MMP-9, MPO and PAI-1 and further, a sensitivity of 80–250,000 pg/ml for sICAM-1, sVCAM-1 and sE-Selectin; with an intra- and interassay CV of 4.5–12.3 % and 8.5–16.3 %, respectively. Plasma glucose was measured using an automated glucose analyser (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured by a chemiluminescent assay (DPC, Los Angeles,

USA) with a sensitivity of 14.3 pmol/l and intra- and interassay CV of 3.7 and 6.7 %, respectively.

### Statistical analysis

All analyses were performed using statistical software (SPSS, version 14; Woking, UK). Variables that were not normally distributed were log transformed. Differences between groups were calculated using a Student's *t*-test for independent samples. A Pearson's correlation analysis was used to analyse bivariate relationships between endotoxin and the various markers of inflammation and vascular injury. Data were expressed as mean  $\pm$  SD. A *p*-value  $< 0.05$  was considered statistically significant.

### Results

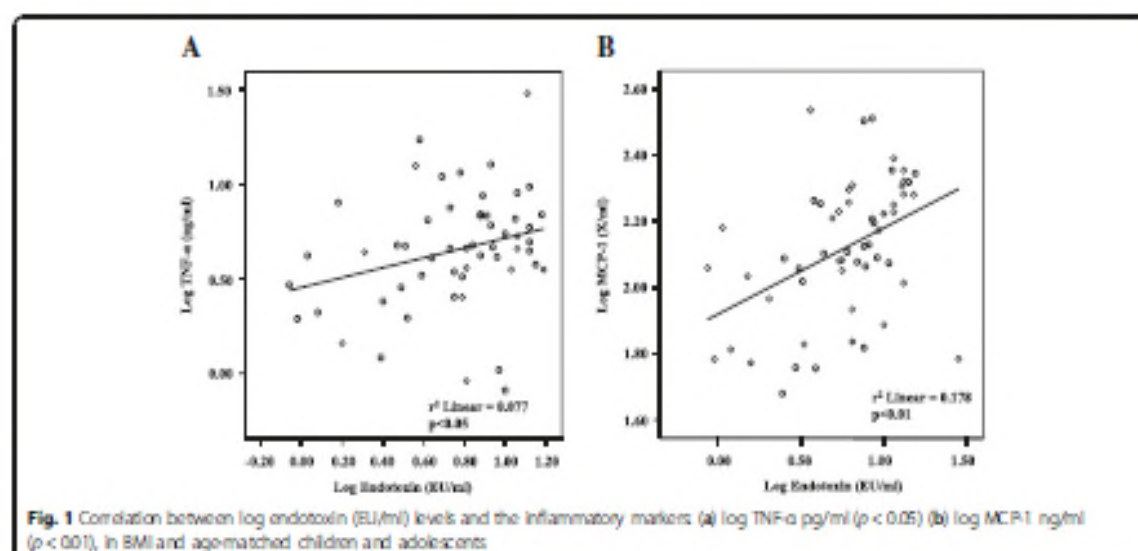
#### Effect of gender on biomarkers of inflammation

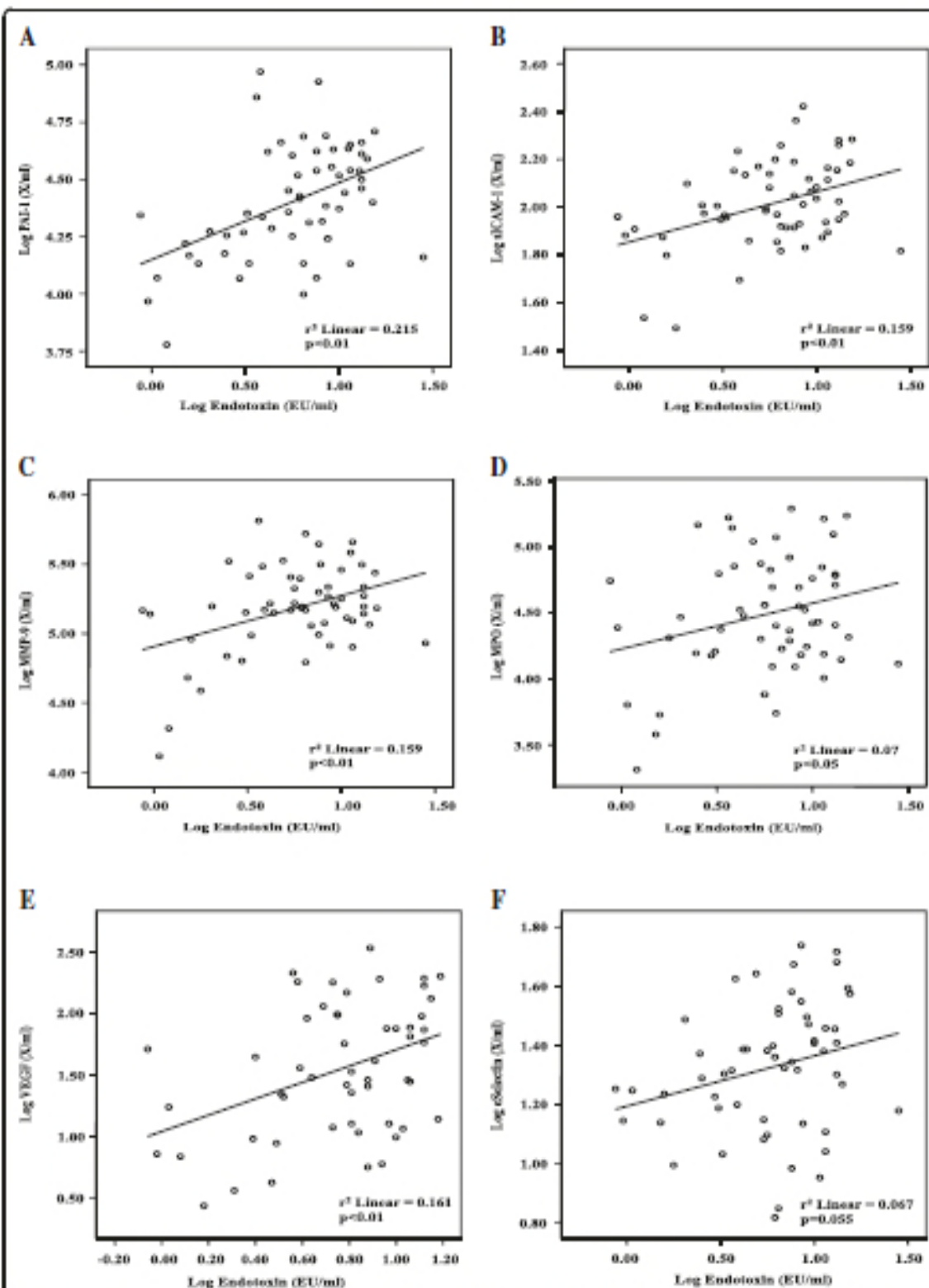
From this cohort, biochemical analysis was performed on 24 male subjects and 36 female subjects with matching BMI and age. Clinical and biochemical characteristics of male and female obese subjects are provided in Table 1. No significant gender differences were observed in HOMA-IR, blood pressure or several markers of inflammation and CV injury.

#### Correlation of endotoxin with biomarkers of inflammation and CVD in childhood & adolescent obesity

In this study circulating endotoxin concentrations significantly and positively correlated with TNF- $\alpha$  ( $p < 0.05$ ,  $r^2 = 0.077$ ) and MCP-1 ( $p < 0.01$ ,  $r^2 = 0.178$ ) (Fig. 1a, b). However, no significant correlation was noted between circulating endotoxin levels and CRP ( $p = \text{NS}$ ,  $r^2 = -0.069$ ).

With regards to CVD risk markers, circulating endotoxin concentrations further correlated with several





**Fig. 2** Correlation between log endotoxin (EU/ml) levels and the following markers of CVD: (a) log PAI-1 (ng/ml) ( $p < 0.05$ ), (b) log sICAM-1 (ng/ml) ( $p < 0.05$ ), (c) log MMP-9 (pg/ml) ( $p < 0.05$ ), (d) log MPO (pg/ml) ( $p < 0.05$ ), (e) log VEGF (pg/ml) ( $p < 0.05$ ) and (f) log eSelectin (X/ml) ( $p < 0.05$ ) in BMI and age-matched children and adolescents



parameters of atherogenesis and vascular injury; these included PAI-1 ( $p < 0.01$ ,  $r^2 = 0.215$ ), sICAM-1 ( $p < 0.01$ ,  $r^2 = 0.159$ ), MMP-9 ( $p < 0.01$ ,  $r^2 = 0.159$ ), MPO ( $p < 0.05$ ,  $r^2 = 0.07$ ) and VEGF ( $p < 0.01$ ,  $r^2 = 0.161$ ) (Fig. 2a–f). No significant correlation was observed between endotoxin and sE-Selectin levels ( $p = 0.055$ ,  $r^2 = 0.067$ ) or sVCAM-1 levels ( $p = N.S.$ ,  $r^2 = 0.054$ ).

Additional analysis of serum concentration data revealed that circulating endotoxin levels positively correlated with systolic blood pressure ( $p < 0.05$ ;  $r^2 = 0.155$ ;  $n = 37$ ) (Fig. 3a) and diastolic blood pressure ( $p < 0.05$ ;  $r^2 = 0.083$ ;  $n = 51$ ) (Fig. 3b). Endotoxin was higher in males than females as a direct comparison (Fig. 4).

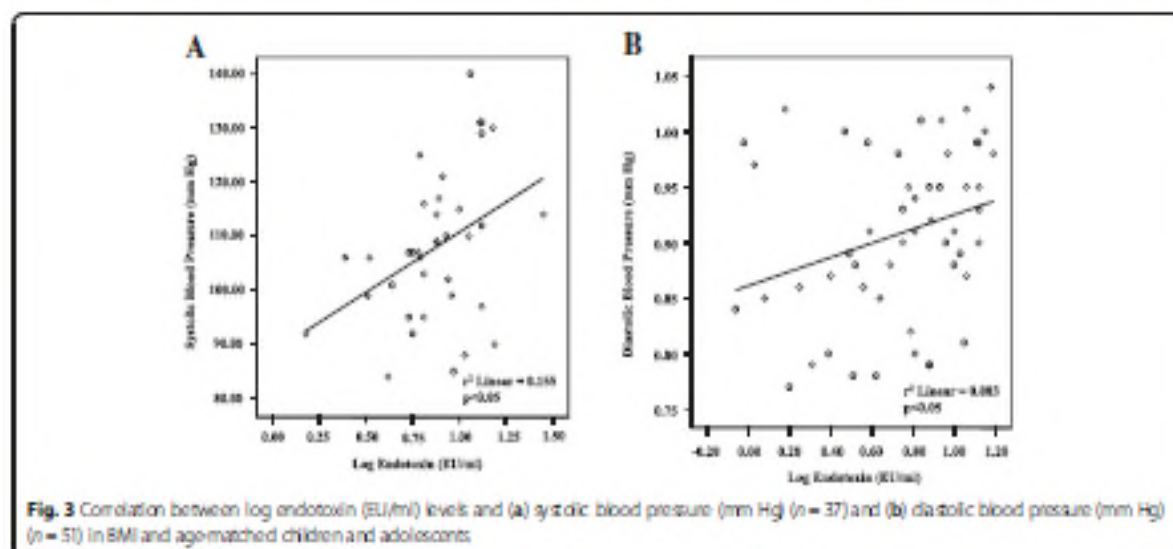
## Discussion

These current studies highlight that in childhood obesity circulating endotoxin is significantly correlated with pro-inflammatory markers, TNF- $\alpha$ , MCP-1, as well as biomarkers of atherogenesis and vascular injury including PAI-1, sICAM-1, MMP-9 and MPO, VEGF. Furthermore, data analysis also determined that circulating endotoxin levels are positively correlated with both systolic and diastolic blood pressure in children with obesity. Taken together these findings also indicate that the presence of metabolic endotoxaemia, which appears to occur in childhood, correlates with pathogenic pro-inflammatory factors in a similar manner to that noted in adults with metabolic disease [14, 15, 17, 21, 22]. These studies also appear to suggest that the raised endotoxin levels in childhood are coupled to a noted high disease risk profile and blood pressure, which together ultimately could lead to an earlier life progression of CVD. Endotoxin may also account for, in part, the continual pro-inflammatory state experienced in obese children [28–31].

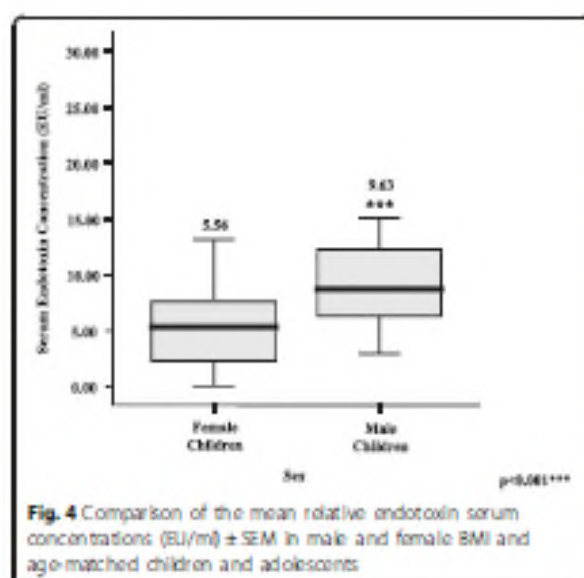
These studies also observed for the first time a noted significant rise in circulating endotoxin in boys compared with girls. Whilst this was not the aim of the study these findings appear consistent with gender-specific effects noted in adults; maintained across several ethnicities [19]. Prior studies in adults suggest a higher endotoxin-induced pro-inflammatory cytokine release in men than women, although this study in children did not identify this gender specific aspect [32, 33]. Such a disparity in endotoxin-induced pro-inflammatory cytokine release between the children and adults with obesity may arise, in part, due to the difference in exposure time to endotoxin; which in obese adults may give rise to long-term damaging inflammatory change leading to T2DM [26, 34, 35].

These studies also suggest that changes in inflammation, vascular dysfunction, and blood pressure in childhood obesity may arise beyond the known impact of the cardiometabolic lipid profile [29, 30, 32, 37, 38]. A key mediator to increase disease risk arise from circulating commensal bacterial endotoxin, derived from the gastrointestinal-tract eliciting a pro-inflammatory response in prior childhood and adolescent obesity studies [36, 39, 40]. Additionally, adult studies highlight that diets high in fat and/or processed meat appear to raise endotoxin levels further, whilst dairy products and other food combinations may reduce endotoxin levels and inflammation [18, 41–43]. As such further future insight into examining the impact of diet on endotoxin levels in children with obesity may highlight important interventions to reduce the long-term health risk [14, 40, 44–46].

Several risk factors in childhood have been proposed to predict the later development of CVD including obesity, hypertension and endothelial dysfunction promoting atherogenesis and thrombosis [47–50]. In our cohort of







obese children and adolescents, several biomarkers of vascular injury and endothelial dysfunction, including PAI-1, sICAM-1, MMP-9, MPO and VEGF, were significantly and positively correlated with circulating endotoxin concentrations. The observed pro-inflammatory biomarker risk profile in the obese children appears to be similar to both what has been identified in adults with CVD as well as studies comparing obese and lean children [50, 51].

Hypertension has been identified as a key risk factor for atherogenesis and vascular injury, and this study noted a positive association between endotoxin with systolic and diastolic blood pressure. As such a diet that raises endotoxin levels would appear to also increase blood pressure. However this is a cross-sectional study, which therefore cannot determine a causal relationship, although previous studies have demonstrated that mice fed a continuous endotoxin bolus, exhibited a subsequent increase in vascular dysfunction [52, 53]. This is consistent with the concept that an endotoxin-induced inflammatory response in childhood obesity, may consequently contribute to an accelerated risk of CVD in later life. Furthermore the noted correlations between circulating endotoxin and biomarkers of endothelial dysfunction, in this childhood obesity study, could also promote a cluster of these pro-atherogenic factors contributing to accelerated atherosclerosis, arterial stiffness and CVD in later life [37, 54–57].

This study had some limitations, namely: the cross-sectional design which did not allow causal determinations and a limited sample size indicating modest correlations. Further, the lack of gender-effects on the correlations between endotoxin and the pro-inflammatory factors may also be considered a reflection of the limited cohort size. Thus, future studies exploring further aspects of these observations in larger cohorts are warranted.

## Conclusion

In summary, this study highlights the relationship between endotoxin and several inflammatory and CVD risk biomarkers, particularly as an early player in obesity-related inflammatory disorders during childhood and adolescence. Furthermore, even at an early age in the obese state, female subjects exhibited lower endotoxin levels than their male counterparts, suggesting a more favourable metabolic profile. This may manifest in later life as delayed CVD mortality and morbidity for females in contrast to males. Finally, among the inflammatory markers evaluated in this study, endotoxin may serve as a potential mediator of sub-clinical inflammation in childhood and adolescent obesity as noted in adult studies.

## Abbreviations

BMI: Body mass index; CRP: c-reactive protein; CVD: Cardiovascular disease; HDL: High density lipoprotein; HOMA-IR: Homeostatic model assessment of insulin resistance; ICAM-1: Interleukin adhesion molecule type-1; IL: Interleukin; LDL: Low density lipoprotein; MCP-1: Monocyte chemoattractant protein-1; MMP-9: Matrix metalloproteinase-9; MPO: Myeloperoxidase; PAI-1: Plasminogen activator inhibitor - 1; sICAM-1: Soluble intercellular adhesion molecule type-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; T2DM: Type 2 diabetes mellitus; TLR4: Toll-like receptors; TNF- $\alpha$ : Tumour necrosis factor- $\alpha$ ; VCAM-1: Vascular cell adhesion molecule type-1; VEGF: Vascular endothelial growth factor.

## Competing interests

The authors declare they have no competing interests.

## Authors' contributions

MV, CK, SA conducted the experimental work for the clinical samples and data analysis as well as writing and revision of manuscript. Q, LG undertook all work leading to ethical approval of the project, selection and recruitment of patients, taking the clinical history and blood sample collection as well as input into the design of the experiment and manuscript revision. SK inputted into the design, data interpretation and coordination of the study. PGM lead the experimental design, supported data analysis, data interpretation, in addition to writing the manuscript. All authors read and approved the final manuscript.

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## Article: Pathophysiology

# Impact of acute hyperglycaemia on endothelial function and retinal vascular reactivity in patients with Type 2 diabetes

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### Abstract

**Aim** In diabetes, endothelial dysfunction and an altered retinal blood flow have been reported and precede overt macrovascular and microvascular disease. Furthermore, an association between postprandial hyperglycaemia, retinopathy and cardiovascular disease has been observed.

**Methods** Endothelial function and retinal vascular reactivity have been measured in baseline conditions in 10 healthy control subjects and 21 patients with Type 2 diabetes. In the patients with Type 2 diabetes, endothelial function and retinal vascular reactivity have been also measured every hour, for 4 h, during an oral glucose tolerance test. Endothelial function has been evaluated by measuring flow-mediated vasodilation of the brachial artery, while retinal vascular reactivity has been measured using a retinal vessel analyser, during a flicker.

**Results** At 1 and 2 h after glucose ingestion, endothelial function decreased ( $P < 0.05$ ), while retinal vascular reactivity increased, even at 3 h ( $P < 0.05$ ), vs. the baseline values.

**Conclusion** Our data highlight that acute hyperglycaemia impacts on endothelial function simultaneously at both macrovascular and at microvascular levels, inducing functional change, which could contribute towards explaining the clinical evidence of a strong association between postprandial hyperglycaemia, cardiovascular disease and retinopathy.

Diabet. Med. 28, 450–454 (2011)

**Keywords** acute hyperglycaemia, endothelial function, retinal vasoreactivity

### Introduction

Current evidence supports the concept that hyperglycaemia significantly contributes to the development of both cardiovascular and microvascular complications of Type 2 diabetes [1]. Diabetic retinopathy is a highly specific microvascular complication of diabetes and the leading cause of blindness in industrialized countries [2].

Endothelial dysfunction is considered a key factor in the development of both cardiovascular disease and retinopathy [3,4]. However, whilst it is widely accepted that this condition produces a reduced circulation at the level of large arteries [3], the

data regarding the retinal circulation are still controversial. In patients with diabetes with absent or mild diabetic retinopathy, increased retinal blood flow, more than a decrease, has been reported [4]. Grunwald *et al.* [5] investigated retinal haemodynamics in the macular microcirculation using the blue field stimulation technique and in the major retinal veins by a combination of bidirectional laser Doppler velocimetry and monochromatic fundus photography. Progression of diabetic retinopathy was assessed from fundus photographs taken at baseline and at the end of the study. The results suggested that increased flow in the macular microcirculation may be associated with progression of retinopathy, thus supporting the hypothesis that increased blood flow may play a role in the development of diabetic microangiopathy.

It has been widely reported that an acute increase of glycaemia produces an endothelial dysfunction at the macrovascular level in

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both normal and patients with diabetes [6]. The data on the effect of acute hyperglycaemia on retinal blood flow are still controversial. It has been reported that acute hyperglycaemia both does not affect retinal circulation [7] or reduces it [8], and that postprandial hyperglycaemia reduces oscillatory potentials in subjects with Type 2 diabetes mellitus [9]. Retinal function can be measured by electroretinogram. With this methodology, the retinal oscillatory potential is evaluated by two waves: the a-wave, used as an index of outer retinal function; and the b-wave, as an index of inner retinal function. The abnormalities in the oscillatory potentials seem to reflect severe disturbances in the retinal circulation. A correlation appears to exist between severely reduced oscillatory potentials and a circulatory deficiency in the retina [9].

The aim of this study was to explore the effect of acute hyperglycaemia, during an oral glucose tolerance test, simultaneously at the level of macro- and retinal circulation in patients with Type 2 diabetes.

## Patients and methods

Twenty-one patients with Type 2 diabetes (duration of disease less than 7 years, 17 patients on oral agents and diet and four on diet only) and 10 healthy control subjects matched for age and BMI were recruited into the study. The clinical characteristics of the control group and the group with diabetes are reported in Table 1.

All subjects were non-smokers. They had a normal resting electrocardiogram, renal function tests and blood pressure. They had no history of any vascular disease or retinopathy. They were not taking aspirin, statins or supplemental vitamins at the time of recruitment into the study. These drugs can influence endothelial

function [10,11]. All subjects then had their height, weight, abdominal circumference and BMI measurements taken using the same standard equipment. Blood samples were taken either from the right or left antecubital vein in a sitting position. Blood pressure was checked with a blood pressure monitor on the left arm and rechecked after 2 min. Finally a 12-lead electrocardiogram was performed, using the same machine for the duration of study. Ethical approval was obtained for the conduct of the study from the Coventry Research Ethics committee, UK.

## Study protocol

In patients with diabetes and control subjects, vascular function tests, including flow-mediated dilatation at the level of the brachial artery and retinal vessel reactivity, were performed under basal fasting conditions. In patients with Type 2 diabetes, at timed intervals before and after a standard oral glucose tolerance test, both flow-mediated dilatation and retinal vessel reactivity were evaluated. Blood samples for assessing glycaemia were drawn at 0 (fasting state), 1, 2, 3 and 4 h post-oral glucose tolerance test (75 g glucose).

## Flow-mediated dilatation

Endothelial function at the macrovascular level was evaluated by measuring flow-mediated dilatation of the brachial artery. The examination was carried out in a temperature- and light-controlled room on subjects who were lying comfortably, flat on a couch. Brachial arteries in this study were imaged with a standard ultrasound system (VIDI 7 ECHO machine, System V; GE Vingmed, Horten, NO, USA) connected with a 12-MHz linear transducer probe. The ultrasound system was connected to a personal computer equipped with a frame grabber and artificial neural network wall detection software (vessel image analysis) [12]. Brachial artery flow-mediated dilatation (LOGIQ e<sup>®</sup> system; GE Vingmed Horten, NO, USA) was determined using protocol similar to published studies [12].

At the end of the study each day, 250 µg of sublingual glyceryl trinitrate was administered in order to assess endothelium independent vasodilatation [11].

The intraobserver variability for repeated measurements of resting arterial diameter was  $0.02 \pm 0.02$  mm.

## Retinal vessel reactivity measurements

Retinal vessel reactivity [13] was measured using a retinal vessel analyser (RVA; Imedos GmbH, Jena, Germany) [14]. The basic components of this system consisted of a fundus camera with a field of 50° (model FF 450; Zeiss, Jena, Germany), a video camera, a real-time monitor and a computer with image-analysis software for the accurate determination of retinal arterial and venous diameter.

All subjects had 1% Tropicamide (Alcon, Cork, Ireland) applied topically to the right eye. This was performed 15–30 min before the actual scanning for pupillary dilatation. The subjects

**Table 1** Baseline characteristics of controls and patients with diabetes

	Control subjects	Patients with Type 2 diabetes
Sex	7 male, 3 female	12 male, 9 female
Age (years)	39.8 ± 3.6	46.4 ± 2.1
BMI (kg/m <sup>2</sup> )	25.4 ± 1.0	30.1 ± 1.1*
Fasting plasma glucose (mmol/l)	5.2 ± 0.2	7.8 ± 0.4†
HbA <sub>1c</sub> (%)	5.8 ± 0.1	7.8 ± 0.3†
HbA <sub>1c</sub> (mmol/mol)	40 ± 0.7	55 ± 2.1†
Resting diastolic blood pressure (mmHg)	71.6 ± 2.4	76.1 ± 1.7
Total cholesterol (mmol/l)	5.1 ± 0.3	4.5 ± 0.2*
Triglycerides (mmol/l)	1.3 ± 0.2	1.6 ± 0.1
HDL cholesterol (mmol/l)	1.4 ± 0.1	1.2 ± 0.1
LDL cholesterol (mmol/l)	3.1 ± 0.2	2.5 ± 0.2
FMD (%)	9.8 ± 1.6	2.5 ± 0.4†
RVR (%)	3.2 ± 0.5	1.4 ± 0.4*

Data are expressed as means ± SEM.

\**P* < 0.05 vs. healthy controls.

†*P* < 0.001 vs. healthy controls.

FMD, flow-mediated dilatation; RVR, retinal vessel reactivity.



were seated comfortably for few minutes, with the height adjusted so that the subject was extremely stable and comfortable for the duration of the study. The left eye was covered to improve fixation of the right eye during imaging with the fundus camera. The image of the retina was adjusted on the screen of the real-time monitor after adjusting the camera to the dilated pupil, and a suitable area in the retina was picked.

The best area for scanning was picked based on the course of artery and vein, distance from the fundus and lack of branches. The superior or inferior quadrant of the temporal region was the usual area picked. Blood pressure and pulse-rate readings were taken before the start of the scan. The recordings were also stored independently and could be evaluated offline. After obtaining a clear fundus image with good contrast and no reflections, the scanning was started according to a fixed protocol [15]. The fundus of the eye was examined under green light. After the baseline recording of 50 s, where both artery and vein in the chosen segment were continuously monitored, three flicker periods of 20 s each were applied, interrupted with 80 s of still illumination. The flickers were created by interrupting the green light. The maximum vasomotion in response to flicker stimulation was measured in two chosen segments of the major temporal inferior branch of the arteriole and venule. The point of maximum dilation was determined for the duration of each flicker stimulation as the highest 1-s mean vessel diameter during the period of 50 s after the beginning of the corresponding flicker stimulus [15]. Three points of maximum dilation were obtained in all measuring sites (proximal and distal in arterioles and venules) and expressed as a percentage of the corresponding first baseline value.

#### Biochemical and physical parameters

Blood samples were drawn at 0 (fasting state), 1, 2, 3 and 4 h for the assay of glucose using the glucose oxidase method.

#### Power calculations

To ensure that the study design had reasonably high statistical power to detect the smallest difference of group means for flow-mediated dilatation, the power calculation was performed in G\*Power 3.0.5 ([http://download.cnet.com/G-Power/3000-2054\\_4-10647044.html](http://download.cnet.com/G-Power/3000-2054_4-10647044.html)). The acceptable power was set conventionally to 0.80 ( $P = 0.20$ ) and statistical significance level ( $P$ ) was 0.05 (two-tailed). No dynamic data were available for retinal vessel reactivity, therefore the sample size was calculated for flow-mediated dilatation based on our previous study [11]. The estimated power was 0.92, revealing that the sample size of 20 subjects was adequate to determine whether the response of endothelium to glucose during an oral glucose tolerance test is significant or not.

#### Validity of tests

All the above values were confirmed by the high levels of validity measurements recorded for each scan. Using the flow-mediated

dilatation software, the obtained values were corroborated by the low levels of noise recorded for each scan. According to the manufacturer of the software program, a noise level of less than 40% is considered as valid for the results obtained [12]. This confirms the validity of the values obtained at each time point for the study. According to the manufacturer of the RVA software program, a validity measurement of more than 40% (valid cycles) was considered to be a reliable indicator of the accuracy of the results obtained [14]. This confirms the validity of the retinal vessel reactivity values obtained at each time point for the study.

#### Statistical analysis

Statistical analysis for this study was performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA).

Initially, the Kolmogorov-Smirnov test was used to determine whether variables had normal distribution. Baseline data comparisons between patients with Type 2 diabetes and the control subjects without diabetes were performed using unpaired Student's *t*-test or the Wilcoxon signed rank test, where appropriate.

In addition, for both flow-mediated dilatation and retinal vessel reactivity evaluation during the oral glucose tolerance test, non-parametric tests, Kruskal-Wallis one-way ANOVA and Wilcoxon signed ranks test, were used. Statistical significance was defined as  $P < 0.05$ . Data are expressed as mean  $\pm$  SEM.

#### Results

As reported in Table 1, seven male and three female control subjects were recruited, while 12 male and nine female patients with Type 2 diabetes participated. The ages of the control subjects and the patients with Type 2 diabetes were  $39.8 \pm 3.6$  and  $46.4 \pm 2.1$  years, respectively, and the BMI measurements for the control subjects and the patients with Type 2 diabetes were  $25.4 \pm 1.0$  and  $30.1 \pm 1.1$  kg/m<sup>2</sup>, respectively. Baseline flow-mediated dilatation and retinal vessel reactivity were significantly lower in patients with Type 2 diabetes compared with the control subjects without diabetes (Table 1).

As expected, during the oral glucose tolerance test, glycaemia in patients with diabetes rose rapidly by 3 h and returned to basal values at the end of the study (Fig. 1).

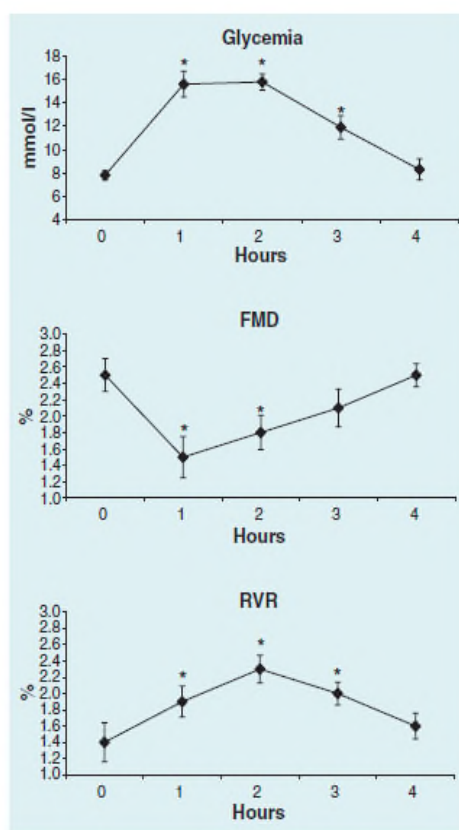
At 1 and 2 h post-oral glucose tolerance test, the mean flow-mediated dilatation was significantly reduced ( $P < 0.05$ ). By 3 h after the oral glucose tolerance test, the flow-mediated dilatation slowly started to recover. By 4 h after the oral glucose tolerance test, the flow-mediated dilatation showed recovery. This level was comparable with the baseline level.

The flow-mediated dilatation dynamic change to the oral glucose tolerance test is shown in Fig. 1.

Endothelial independent vasodilatation was not affected in this experiment (baseline  $12.3 \pm 4.4$  vs.  $12.2 \pm 4.0\%$ ).

Retinal vessel reactivity was represented as percentages similar to flow-mediated dilatation brachial artery measurements.





**FIGURE 1** Glycaemia, flow-mediated dilatation (FMD) and retinal vessel reactivity (RVR) during the oral glucose tolerance test in patients with Type 2 diabetes. \* $P < 0.05$  vs. baseline.

Baseline values are calculated using the RVA software during the time when no flicker was given but the vessels were being monitored.

Similarly, but in contrast to flow-mediated dilatation, at 1 and 2 h, and also at the 3-h post-glucose ingestion, the mean retinal vessel reactivity significantly increased ( $P < 0.05$ ) and was back to baseline levels at the 4 h time point. These data are also reported in Fig. 1.

## Discussion

In this study, we report, for the first time, that an acute increase of glycaemia simultaneously induces an alteration at the level of macro-circulation, in the forearm, and in the retina in patients with well-controlled Type 2 diabetes. We further confirm that, in basal, fasting conditions, both flow-mediated dilatation and retinal vessel reactivity are decreased in patients with Type 2 diabetes compared with control subjects [16,17].

While there is a general agreement that acute hyperglycaemia induces an endothelial dysfunction at the level of macro-circulation [3], data on the retinal circulation are controversial.

No effect or worsening action of hyperglycaemia on retinal circulation have both been reported [7,8].

Moreover, hyperglycaemia in the postprandial state reduces oscillatory potentials [9]. In our opinion, these discrepancies could be explained by the selection of patients and, in particular, by the type of concomitant therapies. It has been suggested, and reasonably so, that nitric oxide production is involved in the effect of hyperglycaemia, not only at the level of forearm circulation but also at the level of the retinal circulation [17]. Several compounds largely used in patients with diabetes, such as ACE inhibitors, AT-1 blockers and statins, can interfere with nitric oxide action [6]. In previous studies, for the most part these factors have not been taken in account, whereas none of our patients were treated with these therapies during the study.

Diabetic retinopathy is a highly specific microvascular complication and it is a result of microvascular retinal changes ultimately leading to parenchymal changes. The two most important features of retinopathy include microvascular calibre changes and microvascular dynamic reactivity to stress, particularly to physiologic states such as hyperglycaemia, especially in people with diabetes. Apart from obvious parenchymal changes, these markers are highly significant. In a population-based prospective study, it was noted that retinal vascular calibre predicted cardiovascular death, independent of traditional cardiovascular risk factors in men and women aged 49–75 years [18]. Furthermore, generalized narrowing of the retinal arterioles has now been associated with incident stroke, coronary artery disease and hypertension, independent of other risk factors [19].

The evidence that acute hyperglycaemia during an oral glucose tolerance test induces simultaneously an endothelial dysfunction in the forearm and vasodilatation in the retina, in our opinion, deserves attention. Endothelial dysfunction is considered an independent risk factor for a future cardiovascular events [3], while increased blood flow in the retina is considered an early risk factor for the development of retinopathy [4].

Postprandial hyperglycaemia is an important contributing factor in the development of cardiovascular disease in people with diabetes [19]. Intervention studies are controversial [21,22]; however, some have supported the relevance of postprandial hyperglycaemia in the development of cardiovascular disease, including silent myocardial infarction [19]. There is now a strong clinical association between retinopathy and cardiovascular disease in diabetes, with retinopathy associated with an approximate twofold increased risk for cardiovascular disease and all-cause mortality in individuals with Type 2 diabetes [23]. Postprandial hyperglycaemia is now being considered an independent risk factor for the development of retinopathy in diabetes [24]. It is also worthy of interest, the recent demonstration that 'post-challenge' glucose levels measured during an oral glucose tolerance test might be used as a predictor of 'postprandial hyperglycaemia' [25]. With this backdrop, the study points to our finding that the macro-circulation and the retinal circulation simultaneously respond to an acute increase of glycaemia. This also explains why oral glucose tolerance testing



in normal subjects was not performed: by definition, the values of glycaemia would have been in the normal range or slightly increased only in the first hour. In the absence of a significant increase of glycaemia, it would have been difficult to detect any change in endothelial function.

We do acknowledge the fact that, during an oral glucose tolerance test, apart from glucose, changes in the levels of other hormones and insulin could be involved in endothelial influence. But, we do believe that these effects should be similar in both groups and therefore unlikely to explain the difference between groups. This finding could advance our current opinion and help to better explain the relationship between increased cardiovascular disease risk and retinopathy changes.

### Competing interests

Nothing to declare.

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# High Fat Intake Leads to Acute Postprandial Exposure to Circulating Endotoxin in Type 2 Diabetic Subjects

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**OBJECTIVE**—To evaluate the changes in circulating endotoxin after a high-saturated fat meal to determine whether these effects depend on metabolic disease state.

**RESEARCH DESIGN AND METHODS**—Subjects ( $n = 54$ ) were given a high-fat meal (75 g fat, 5 g carbohydrate, 6 g protein) after an overnight fast (nonobese control [NOC]: age  $39.9 \pm 11.8$  years [mean  $\pm$  SD], BMI  $24.9 \pm 3.2$  kg/m<sup>2</sup>,  $n = 9$ ; obese: age  $43.8 \pm 9.5$  years, BMI  $33.3 \pm 2.5$  kg/m<sup>2</sup>,  $n = 15$ ; impaired glucose tolerance [IGT]: age  $41.7 \pm 11.3$  years, BMI  $32.0 \pm 4.5$  kg/m<sup>2</sup>,  $n = 12$ ; type 2 diabetic: age  $45.4 \pm 10.1$  years, BMI  $30.3 \pm 4.5$  kg/m<sup>2</sup>,  $n = 18$ ). Blood was collected before (0 h) and after the meal (1–4 h) for analysis.

**RESULTS**—Baseline endotoxin was significantly higher in the type 2 diabetic and IGT subjects than in NOC subjects, with baseline circulating endotoxin levels 60.6% higher in type 2 diabetic subjects than in NOC subjects ( $P < 0.05$ ). Ingestion of a high-fat meal led to a significant rise in endotoxin levels in type 2 diabetic, IGT, and obese subjects over the 4-h time period ( $P < 0.05$ ). These findings also showed that, at 4 h after a meal, type 2 diabetic subjects had higher circulating endotoxin levels (125.4%†) than NOC subjects ( $P < 0.05$ ).

**CONCLUSIONS**—These studies have highlighted that exposure to a high-fat meal elevates circulating endotoxin irrespective of metabolic state, as early as 1 h after a meal. However, this increase is substantial in IGT and type 2 diabetic subjects, suggesting that metabolic endotoxemia is exacerbated after high fat intake. In conclusion, our data suggest that, in a compromised metabolic state such as type 2 diabetes, a continual snacking routine will cumulatively promote their condition more rapidly than in other individuals because of the greater exposure to endotoxin.

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**S**tudies examining the interrelationships between adipose tissue, inflammation, and insulin resistance appear key to understanding type 2 diabetes risk (1,2). It is known that low-grade chronic systemic inflammation contributes to this risk, which appears altered by several factors such as increasing age, sex, ethnicity, genetics, and dietary influences. However, systemic inflammation appears to persist

in type 2 diabetic subjects, despite medication, while the mechanisms and mediators of this continual inflammation appear less clear. Evidently, adipose tissue accumulation has a significant impact on disease risk and inflammation in type 2 diabetes but may merely act in response to systemic primary insults (3–9).

One potential cellular mechanism for increased inflammation may arise through

activation of the innate immune system in human adipose tissue (10–13). Previous studies have shown that increased activation of the innate immune pathway may arise through excess circulating gut-derived bacteria, known as lipopolysaccharide (LPS) or endotoxin, which represents the outer cell wall membrane of gram-negative bacteria (10,11,14–17). Our previous work has shown that endotoxin has an immediate impact on the innate immune pathway in human adipose tissue, acting via key receptors known as the Toll-like receptors, which recognize antigens, such as the LPS component, to initiate an acute-phase response to infection (8,10). Stimulation of the Toll-like receptors leads to intracellular activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), a key transcription factor in the inflammatory cascade that regulates the transcription of numerous proinflammatory adipokines (9,10). Therefore, in vitro endotoxin may act as a mediator of inflammation through activation of NF- $\kappa$ B, leading to a rapid response within adipose tissue that may be exacerbated by increased adipose tissue mass (18–22).

However, clinical studies have also implicated gut-derived endotoxin as a “primary insult” to activate the inflammatory state, contributing to metabolic disease, with current cross-sectional data showing elevated systemic endotoxin levels in conditions of obesity, type 2 diabetes, coronary artery disease, and fatty liver disease (8,10,11,14–17). Within these studies, circulating endotoxin is observed to be positively associated with waist circumference, waist-to-hip ratio, insulin levels, inflammatory cytokines and lipids, including total cholesterol, triglycerides (TGs), and LDL cholesterol, and negatively associated with HDL cholesterol (8,10,11,14–17). The combined importance of dietary lipids and LPS in determining inflammatory risk may arise, since endotoxin has a strong affinity for chylomicrons (lipoproteins that transport dietary long-chain saturated fatty acids [SFAs] through the gut wall) as endotoxin crosses the gastrointestinal mucosa (23–25). As such, atherogenic and inflammatory risk may arise through a

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combination of dietary lipoprotein patterns and an increase in circulating endotoxin, exacerbated by feeding patterns (26,27). Therefore, altering the lipid profile through dietary intervention may reduce endotoxin and the arising inflammatory response. Recent human studies have explored dietary effects of a high-SFA, high-carbohydrate meal on circulating endotoxin levels in healthy individuals. The findings showed a substantial increase in circulating endotoxin, in subjects given a high-fat meal, in conjunction with markers of inflammation (as noted from mononuclear blood cells) (13,28). Murine studies have also identified an association between endotoxin and insulin resistance, through infusion of endotoxin, with the same effect also noted by a high-fat diet (12), with insulin resistance and weight gain both affecting gut permeability (11,17,28). In studies to date, using either infused endotoxin as a bolus or derived from the gut because of dietary changes, both methods suggest endotoxin has the capacity to affect the inflammatory pathways (28,29). However, it remains to be established whether diets in different metabolic states affect absorption of endotoxin. Also, do such postprandial circulating endotoxin levels correlate with systemic lipid changes postprandially, being compounded in more insulin-resistant states? Therefore, these studies sought to establish whether a high-fat meal increased circulating endotoxin and whether this is altered in different metabolic disease states.

## RESEARCH DESIGN AND METHODS

The study consisted of healthy control subjects ( $n = 9$ ), obese subjects ( $n = 15$ ), and patients with impaired glucose tolerance (IGT) ( $n = 12$ ) and type 2 diabetes ( $n = 18$ ). All subjects with type 2 diabetes were of South Asian origin except one subject, who was Afro-Caribbean. Similarly, all healthy control subjects were South Asian in origin except one, who was Afro-Caribbean.

All subjects were nonsmokers. Screening blood tests were performed for both baseline measurements to qualify for the study, as well as to assess glucose control. Routine blood tests included renal function, glucose, HbA<sub>1c</sub>, and full cholesterol profile. All subjects had their height, weight, abdominal circumference, and BMI measurements taken using standard equipment. Blood samples were taken either from the right or left antecubital vein in a sitting position. Blood pressure was

checked with a blood pressure monitor on the left arm and rechecked after 2 min. Finally, a 12-lead electrocardiogram was performed using the same machine for the duration of the study. Subjects included in the study had a normal resting electrocardiogram, normal renal function tests and blood pressure, and no history of vascular disease. Detailed medical drug histories were taken on medications, and those subjects on medication considered to lead to a change in inflammatory status were excluded, including the thiazolidinediones. Ethical approval was obtained from the local research ethics committee, and all patients gave written consent.

All research subjects ( $n = 54$ ) with and without type 2 diabetes were given a high-fat meal (standardized meal: 75 g fat, 5 g carbohydrate, 6 g protein) after an overnight fast of 12–14 h, as previously described (30). The cohort consisted of nonobese control (NOC) subjects (age  $39.9 \pm 11.8$  years [mean  $\pm$  SD], BMI  $24.9 \pm 3.2$  kg/m<sup>2</sup>,  $n = 9$ ) obese subjects (age  $43.8 \pm 9.5$  years, BMI  $33.3 \pm 2.5$  kg/m<sup>2</sup>,  $n = 15$ ) subjects with IGT (age  $41.7 \pm 11.3$  years, BMI  $32.0 \pm 4.5$  kg/m<sup>2</sup>,  $n = 12$ ), and type 2 diabetic subjects (age  $45.4 \pm 10.1$  years, BMI  $30.3 \pm 4.5$  kg/m<sup>2</sup>,  $n = 18$ ). Blood samples were drawn at baseline (0 h) and postprandially (1, 2, 3, and 4 h), and endotoxin and lipid levels were measured.

## In vivo assessment of the biochemical profile

On the assigned date, fasting blood samples were collected from participating subjects, and lipid profiles and fasting plasma glucose were determined using routine laboratory methods undertaken in the biochemistry laboratory at University Hospital Coventry and Warwickshire. In brief, the routine blood tests included renal function, glucose, HbA<sub>1c</sub>, and a full cholesterol profile (TGs, HDL, and LDL), as noted in Table 2. Insulin measurements were performed by a solid-phase enzyme amplified sensitivity multiplex immunoassay (Millipore, Hertfordshire, U.K.), and glucose was measured by a glucose oxidase method (YSI 200 STAT plus). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated for all patients using the HOMA formula:  $\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{plasma glucose (mmol/L)} / 22.5$ .

## Analysis of circulating endotoxin levels

Serum endotoxin was analyzed using a commercially available QCL-1000 LAL

End Point Assay (Lonza, Allendale, NJ). The assay, and the values given by the manufacturer for intra-assay coefficient of variation (CV) ( $3.9 \pm 0.46$ ) and interassay CV ( $9.6 \pm 0.75$ ), have been validated in our laboratory, as detailed previously (10).

## Statistical analysis

For assessment of the different variables, statistical analysis was undertaken using a paired Student *t* test, for intra-comparison of hourly time points versus baseline, and an unpaired Student *t* test for inter-comparisons. The threshold for significance was  $P < 0.05$ . Data in the text and figures are presented as means  $\pm$  SD or means  $\pm$  SEM. Correlations were determined with a Pearson correlation. Variables with a non-Gaussian distribution were logarithmically or square root transformed, as deemed appropriate, before statistical analysis. All statistics were performed on SPSS version 17.0.

## RESULTS

### Baseline characteristics across groups

Table 1 shows the anthropometric data. Table 2 shows the metabolic baseline and hour time point characteristics of the four groups analyzed in this study. Age did not differ significantly between the groups, whereas BMI was altered across the groups, with the NOC group possessing the lowest BMI ( $24.9 \pm 3.2$  kg/m<sup>2</sup>) (Table 1). The other three groups (obese:  $33.3 \pm 2.5$  kg/m<sup>2</sup>\*\*\*† (increase); IGT:  $32.0 \pm 4.5$  kg/m<sup>2</sup>\*\*\*†, and type 2 diabetic:  $30.3 \pm 4.5$  kg/m<sup>2</sup>\*\*\*† (increase) subjects; *P* value; \*\*\* $P < 0.001$ , \*\* $P < 0.01$ ) differed significantly compared with NOC subjects, whereas the type 2 diabetic group exhibited a significantly lower BMI than the obese cohort ( $P < 0.05$ ). Waist circumference followed a similar pattern to the BMI data. NOC subjects' waist circumference was  $86.9 \pm 8.25$  cm versus obese ( $108.9 \pm 17.9$  cm\*\*\*†), IGT ( $106.4 \pm 10.37$  cm\*\*\*†), and type 2 diabetic ( $100.1 \pm 10.2$  cm\*\*\*†) subjects (Table 1). The mean systolic blood pressure (SBP) levels of the groups were similar across all groups and did not change significantly over the 4-h duration, which was also noted for the diastolic blood pressure (DBP) levels (NOC subjects: SBP  $128.2 \pm 9.7$  mmHg, DBP  $72.3 \pm 10.0$  mmHg; obese: SBP  $128.2 \pm 10.7$  mmHg, DBP  $74.5 \pm 6.9$  mmHg; IGT: SBP  $127.3 \pm 10.1$  mmHg, DBP  $75.1 \pm 8.8$  mmHg; and type 2 diabetic: SBP  $129.1 \pm 10.1$  mmHg, DBP  $74.5 \pm 6.9$  mmHg).



Table 1—Anthropometric data for the different cohorts

	Normal group	Obese group	IGT group	Type 2 diabetic group	P
n	9	15	12	18	
Age (years)	39.9 ± 11.8	43.8 ± 9.5	41.7 ± 11.3	45.4 ± 10.1	NS
Sex (M:F)	6:3	10:5	7:5	11:7	
Ethnicity	Asian, n = 8 Afro-Caribbean, n = 1	Asian, n = 15	Asian, n = 12	Asian, n = 17 Afro-Caribbean, n = 1	
BMI# (kg/m <sup>2</sup> )	24.9 ± 3.2	33.3 ± 2.5	32.0 ± 4.5	30.3 ± 4.5	Normal vs. obese: P < 0.001; normal vs. IGT: P = 0.001; normal vs. T2DM: P = 0.003; IGT vs. obese: NS; IGT vs. T2DM: NS; obese vs. T2DM: P = 0.019
Waist circumference (cm)	86.9 ± 8.25	108.9 ± 17.9	106.4 ± 10.37	100.1 ± 10.2	Normal vs. obese: P = 0.001; normal vs. IGT: P < 0.001; normal vs. T2DM: P = 0.002; IGT vs. obese: NS; IGT vs. T2DM: NS; obese vs. T2DM: NS
Estimated glomerular filtration rate#	105.62 ± 19.71	91.07 ± 17.40	105.92 ± 19.77	92.28 ± 24.36	NS

Data are means ± SD unless otherwise indicated. T2DM, type 2 diabetes. #Data with a non-Gaussian distribution that were transformed before statistical analysis.

As anticipated, the baseline NOC group had significantly lower fasting plasma glucose levels than the type 2 diabetic group, while showing similar glucose levels to the obese and IGT cohorts (NOC:  $4.7 \pm 0.69$  mmol/L vs. type 2 diabetic:  $8.1 \pm 1.8$  mmol/L\*\*\*†; IGT:  $5.6 \pm 1.2$  mmol/L; obese IGT:  $4.9 \pm 0.93$  mmol/L; \*\*\*P < 0.001). HbA<sub>1c</sub> was similar in the obese and NOC groups but was significantly higher in the IGT and type 2 diabetic groups (NOC:  $5.9 \pm 0.31\%$  vs. type 2 diabetic:  $7.5 \pm 1.12\%$ \*\*\*†; IGT:  $6.3 \pm 0.47\%$ \*†; obese IGT:  $5.9 \pm 0.49\%$ ; \*\*\*P < 0.001, \*P < 0.05). Within each cohort, glucose levels were not significantly altered over the 4-h postprandial time period.

The baseline lipid profile across the groups was comparable. Serum endotoxin levels were significantly lower in the baseline NOC group compared with the IGT and type 2 diabetic groups (NOC:  $3.3 \pm 0.15$  endotoxin unit/mL [EU/mL] vs. obese:  $5.1 \pm 0.94$  EU/mL†; IGT:  $5.7 \pm 0.10$  EU/mL\*\*\*†; type 2 diabetic:  $5.3 \pm 0.54$  EU/mL\*†; \*\*P < 0.01, \*P < 0.05; Fig. 1A, Table 2).

#### Postprandial change in endotoxin levels over time in individual groups

Postprandial exposure to a high-fat meal led to a significant rise in endotoxin levels in obese subjects (baseline:  $5.1 \pm 0.94$  EU/mL; 1 h:  $4.2 \pm 0.71$  EU/mL; 2 h:  $6.2 \pm 0.49$  EU/mL; 3 h:  $7.8 \pm 0.76$  EU/mL; 4 h:  $7.7 \pm 0.58$  EU/mL; \*\*\*P < 0.01, \*P < 0.05; Fig. 1A, Table 2);

IGT (IGT: baseline:  $5.7 \pm 0.10$  EU/mL; 1 h:  $5.8 \pm 0.22$  EU/mL; 2 h:  $5.5 \pm 1.0$  EU/mL; 3 h:  $7.4 \pm 0.26$  EU/mL; 4 h:  $7.5 \pm 0.20$  EU/mL; \*P < 0.05, Fig. 1A, Table 2), and type 2 diabetic subjects (baseline:  $5.3 \pm 0.54$  EU/mL; 1 h:  $5.5 \pm 0.44$  EU/mL; 2 h:  $5.8 \pm 0.34$  EU/mL; 3 h:  $9.8 \pm 1.2$  EU/mL; 4 h:  $14.2 \pm 3.0$  EU/mL; \*\*\*P < 0.01, Fig. 1A, Table 2) over the 4-h time period. In the NOC group, whereas there was a rise in circulating endotoxin over the 4-h period, this trend did not reach significance past 1 h (Fig. 1A, Table 2). Fasting endotoxin levels showed a positive correlation with fasting TG levels in the whole cohort ( $r = 0.303$ ,  $P = 0.026$ ). Further examination of this relationship postprandially identified the positive correlation strengthened over time, with the strongest relationship between endotoxin and TG noted at 2 and 3 h, respectively (2-h time point:  $r = 0.531$ ,  $P < 0.001$ ; 3-h time point:  $r = 0.498$ ,  $P < 0.001$ ) with a decline by 4 h after feeding ( $r = 0.434$ ,  $P = 0.001$ ). No further correlations with any other parameters were observed, and those noted were not influenced by age or sex.

#### Postprandial change in endotoxin levels between groups

Fasting endotoxin levels were significantly higher in IGT and type 2 diabetic subjects than in NOC subjects (72.7%\*\*\* and 60.6%\* increase, respectively; \*\*\*P < 0.01, \*P < 0.05, Fig. 2B and C). However, during the postprandial 4-h time period, the circulating endotoxin levels in both obese and IGT

subjects diminished to ~20% higher than that of the NOC (4 h, Fig. 2A and B), whereas circulating endotoxin levels were sustained at significantly higher levels in the type 2 diabetic subjects than in the NOC subjects (4 h, Fig. 2C,  $P < 0.05$ ).

#### Postprandial changes in lipids over time in individual groups

Postprandial exposure to a high-fat meal led to a significant rise in TG levels in NOC, IGT, and type 2 diabetic subjects after 1 h ( $P < 0.05$ , Fig. 1B, Table 2). Although the obese subjects followed the same trend, TG levels were only significantly altered at 2-h post-feeding ( $P < 0.05$ ; Fig. 3, Table 2).

Total cholesterol remained relevantly unaltered over the 4-h period within all four groups (Table 2). In addition, no change was noted in LDL cholesterol and HDL cholesterol for the NOC subjects over the 4-h period (Table 2). LDL cholesterol and HDL cholesterol in the other three groups did show significant individual group changes over time. For all metabolic states, LDL and HDL cholesterol significantly changed (increased and reduced, respectively;  $P < 0.05$ ; Table 2), whereas levels in NOC subjects were not altered.

#### Postprandial changes in lipid levels between groups

Fasting total cholesterol, TG, LDL cholesterol, and HDL cholesterol levels were comparable at baseline within the four groups and did not differ significantly throughout the 4-h duration (Figs. 1B and 3; Table 2).



## Postprandial endotoxin levels

Table 2—Variable data for the different cohorts

	Baseline	1 h	2 h	3 h	4 h
<b>Normal group</b>					
Triglycerides# (mmol/L)	1.1 ± 0.07	1.3 ± 0.08*	1.8 ± 0.08***	2.2 ± 0.2**	2.4 ± 0.27**
Total cholesterol (mmol/L)	4.9 ± 0.95	5.1 ± 0.85	5.1 ± 0.73	5.1 ± 0.94	5.2 ± 0.81
LDL cholesterol# (mmol/L)	3.2 ± 0.04	3.2 ± 0.03	3.1 ± 0.03	2.82 ± 0.04*	2.79 ± 0.04
HDL cholesterol# (mmol/L)	1.1 ± 0.004	1.1 ± 0.004	0.98 ± 0.03	1.06 ± 0.004	1.03 ± 0.008
TNF-α# (pg/mL)	8.0 ± 2.3	8.1 ± 2.2	7.4 ± 2.0	7.4 ± 1.8	8.0 ± 2.0
Leptin# (ng/mL)	16.8 ± 4.8	17.0 ± 5.3*	14.6 ± 4.0*	13.6 ± 3.5*	14.7 ± 4.4
Endotoxin# (EU/mL)	3.3 ± 0.15	4.0 ± 0.17*	4.32 ± 0.19	5.5 ± 0.64	6.3 ± 1.4
<b>Obese group</b>					
Triglycerides# (mmol/L)	1.6 ± 0.08	1.7 ± 0.08	2.3 ± 0.10***	2.7 ± 0.12***	3.0 ± 0.14***
Total cholesterol (mmol/L)	5.3 ± 0.80	5.1 ± 0.67	5.1 ± 0.99	5.3 ± 1.0	5.2 ± 0.76
LDL cholesterol# (mmol/L)	3.56 ± 0.04	3.34 ± 0.02	3.0 ± 0.05***	3.0 ± 0.06***	2.9 ± 0.04***
HDL cholesterol# (mmol/L)	0.92 ± 0.01	0.90 ± 0.01	0.84 ± 0.01*	0.85 ± 0.008**	0.81 ± 0.01***
TNF-α# (pg/mL)	8.4 ± 1.3	8.4 ± 1.4	7.8 ± 1.6	7.5 ± 1.4*	7.5 ± 1.4*
Leptin# (ng/mL)	25.6 ± 3.1	23.3 ± 2.7***	20.7 ± 2.3	21.7 ± 2.6	21.4 ± 2.9***
Endotoxin# (EU/mL)	5.1 ± 0.94	4.2 ± 0.71	6.2 ± 0.49	7.8 ± 0.76**	7.7 ± 0.58**
<b>IGT group</b>					
Triglycerides# (mmol/L)	1.3 ± 0.03	1.5 ± 0.04***	1.9 ± 0.07***	2.5 ± 0.15***	2.5 ± 0.15***
Total cholesterol (mmol/L)	4.9 ± 0.80	4.7 ± 0.74	4.8 ± 0.74	4.8 ± 0.77	4.8 ± 0.77
LDL cholesterol# (mmol/L)	3.24 ± 0.04	3.08 ± 0.03	2.9 ± 0.03**	2.6 ± 0.04**	2.66 ± 0.05**
HDL cholesterol# (mmol/L)	0.88 ± 0.008	0.86 ± 0.008	0.82 ± 0.006***	0.81 ± 0.008***	0.77 ± 0.01***
TNF-α# (pg/mL)	4.4 ± 2.1	4.3 ± 2.0	4.3 ± 2.0	4.1 ± 2.0	4.3 ± 2.1
Leptin# (ng/mL)	37.0 ± 2.7	32.6 ± 2.7***	32.0 ± 2.7*	31.1 ± 3.0**	33.0 ± 3.3**
Endotoxin# (EU/mL)	5.7 ± 0.10	5.8 ± 0.22	5.5 ± 1.0	7.4 ± 0.26*	7.5 ± 0.20*
<b>Type 2 diabetic group</b>					
Triglycerides# (mmol/L)	1.4 ± 0.03	1.6 ± 0.05***	2.2 ± 0.08***	2.8 ± 0.12***	3.1 ± 0.13***
Total cholesterol (mmol/L)	5.0 ± 1.0	4.8 ± 0.95*	4.8 ± 0.91	5.0 ± 1.0	4.9 ± 0.93
LDL cholesterol# (mmol/L)	3.17 ± 0.11	2.9 ± 0.07***	2.6 ± 0.10***	2.4 ± 0.16**	2.4 ± 0.14***
HDL cholesterol# (mmol/L)	1.0 ± 0.02	1.0 ± 0.02	0.94 ± 0.02***	0.92 ± 0.02***	0.86 ± 0.02***
TNF-α# (pg/mL)	8.6 ± 2.0	8.4 ± 2.0	8.4 ± 2.0	8.3 ± 1.8	8.1 ± 2.3
Leptin# (ng/mL)	18.1 ± 3.5	20.0 ± 5.0***	18.7 ± 3.7	18.2 ± 3.4	15.2 ± 3.3
Endotoxin# (EU/mL)	5.3 ± 0.54	5.5 ± 0.44	5.8 ± 0.34	9.8 ± 1.2**	14.2 ± 3.0**

Data are means ± SD. TNF, tumor necrosis factor. #Data with a non-Gaussian distribution that were transformed before statistical analysis. Significant difference compared time points with baseline values. \**P* < 0.05. \*\**P* < 0.01. \*\*\**P* < 0.001.

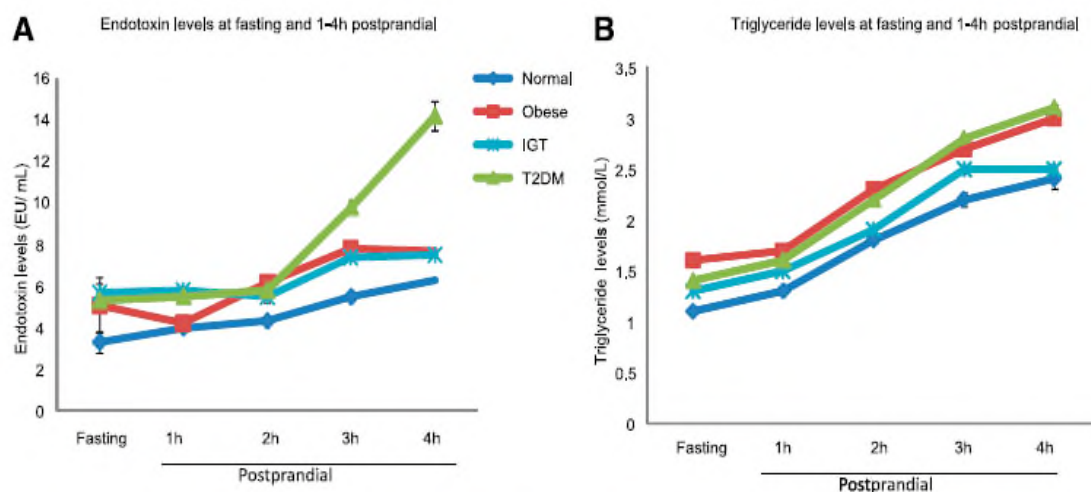
**CONCLUSIONS**—This is the first study to examine the comparative and differential changes in circulating endotoxin after a SFA meal from subjects with and without type 2 diabetes, obesity, or IGT. The novel data highlight that a SFA meal increases circulating endotoxin levels in all subjects irrespective of their metabolic status, although circulating endotoxin shows dramatic postprandial changes in the high-metabolic risk groups. More specific comparative analysis of NOC subjects versus subjects with type 2 diabetes at 4-h postprandial identified that the latter had a mean endotoxin level 125.4% higher than that of NOC subjects. Cumulative data derived from the fasting state and the SFA postprandial state indicate that type 2 diabetic subjects are subjected to 336% more circulating endotoxin than NOC subjects over the 4-h duration. In comparison to other metabolic states, the

obese and IGT subjects were still subjected to 167 and 198.5% more circulating endotoxin than NOC subjects. As such, endotoxin, which is considered a potential mediator of chronic low-grade inflammation, is considerably higher in the state of type 2 diabetes, with implications for a continual inflammatory state, as other articles have observed (15,16,28,29).

While our previous studies have shown significant associations in the fasted state among circulating endotoxin, lipoprotein patterns, and anthropometric data (8,10,11,14–17), these current studies have sought to establish whether endotoxin acutely changes postprandially and whether this is altered by differing metabolic states. By undertaking this, our current studies have highlighted subtle but significant differences in how endotoxin levels change in the postprandial period. After a SFA meal, the NOC

endotoxin levels rose over the 4-h duration, but circulating levels did not increase significantly. In contrast, in the obese and IGT groups, there was a significant rise in endotoxin, which appeared to plateau by 4 h. However, at the 4-h time point, both the IGT and obese groups' endotoxin levels were much lower than those of the type 2 diabetic subjects, since the levels of endotoxin in the type 2 diabetic subjects appeared to still be rising 4 h after a SFA meal. Circulating endotoxin in the type 2 diabetic group, after 4 h, did not appear to normalize, which suggests the cumulative exposure to endotoxin after a high-SFA meal is disproportionately high compared with any other group. Furthermore, in the type 2 diabetic subjects, the rising endotoxin levels may be further compounded by the refeeding stage. These data appear to indicate that a person eating three high-SFA meals each day may encounter endotoxin levels





**Figure 1**—Changes in circulating endotoxin levels (A) and triglyceride levels (B) in NOC, IGT, obese, and type 2 diabetic (T2DM) subjects. Endotoxin and triglyceride levels were measured at baseline and then, after a high-SFA meal, at each hour postprandially over a 4-h duration. Each point on the graph represents the mean value for each cohort ( $\pm$  SEM).

that remain perpetually high, since refeeding may increase the levels. As such, fasted endotoxin data, while important, may appear to miss the daily variation, as feeding data appear to show. The type of meal is clearly important, since previous studies highlight that dietary changes alter circulating endotoxin and influence inflammation, even in healthy subjects (13,28). In addition, recent studies have reported that the simultaneous ingestion of certain “healthy” food groups with saturated fat can negate an increase in circulating endotoxin and the customary inflammatory response (29). Because it is acknowledged that obese and type 2 diabetic subjects tend to eat high SFA without correspondingly high levels of fruit or healthy foods, this diet would clearly affect their endotoxin and inflammatory status (28,31,32). Therefore, a high-SFA intake could represent a continual inflammatory insult for type 2 diabetic subjects, daily.

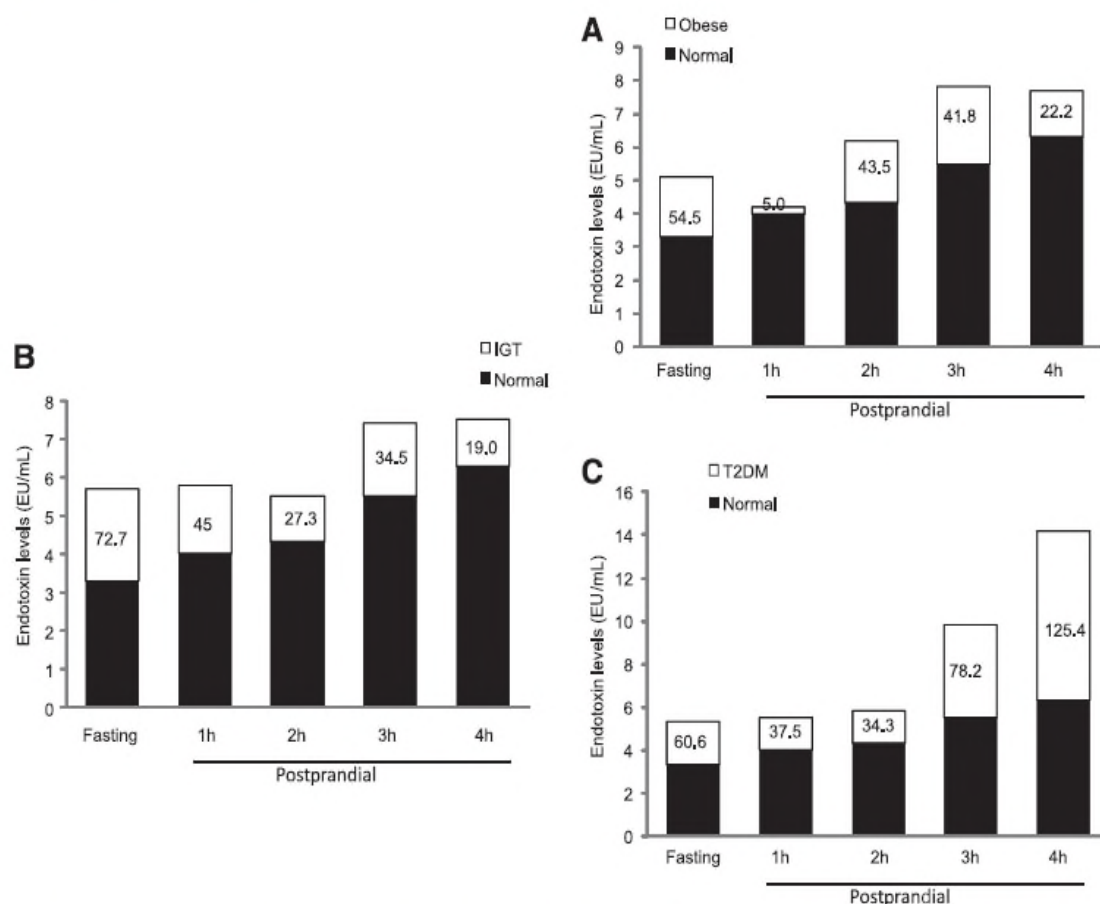
In the obese and IGT groups, the postprandial 4-h endotoxin levels appear to plateau, while still remaining high compared with NOC subjects. Subsequently, another SFA meal may compound the circulating endotoxin levels further within the obese and IGT groups; therefore, the type and frequency of meals may significantly affect the metabolic risk. In addition to the type of meal, the food intake frequency is also relevant, although currently, there are few studies examining the importance of this. Previous studies indicate no difference between a diet based on three meals a day or a diet comprising

smaller meals and snacks, with regard to the long-term effects on glucose, lipid, or insulin responses; although the unknown acute postprandial effects on the inflammatory status may have a more profound long-term impact (32,33). In addition, previous studies have often stressed the division of food intake should be based on individual preference, with no clear recommendations on pattern of food intake. Within type 2 diabetes clinics, the recommendation for patients is to consume five smaller meals per day. This step may reduce the potentially overwhelming orexigenic effects patients might experience with only three meals a day, as well as the potential spikes in insulin, although the data do not necessarily give clear insight into these benefits (32,33). Based on these current studies, more frequent saturated fat exposure may exacerbate both endotoxin and inflammation further. Furthermore, smaller more frequent meals have the potential to allow endotoxin to spike several times a day, thus activating the innate immune system within adipose tissue without desensitization (9,10,28,29). As such, the resulting downstream production of diabetogenic cytokines would be in continuous production, as previous *in vivo* and *in vitro* studies have demonstrated (9,10,17,28,29). The TG levels did not differ significantly across the four groups of subjects at any of the time points; however, the TG levels did increase from baseline to 4 h within each group, in a similar pattern to circulating endotoxin, but most significantly in the metabolic risk subjects

(obese, IGT, and type 2 diabetic), while also demonstrating an association with endotoxin, over the 4-h period (10,16,17). The three different metabolic states showed significantly higher fasting TG levels than NOC subjects, which postprandially became further exacerbated in the obese and type 2 diabetic subjects.

Unsurprisingly, postprandial TG levels increased in a similar pattern to circulating endotoxin, while also demonstrating an association over the 4-h period (10,16,17). The three different metabolic states showed no significant differences in TG levels compared with levels in NOC subjects. However, the significant correlation between fasting TG and endotoxin levels confirms previous studies in which an association between these two metabolic parameters had been observed (10,16,17). Our data indicated that the association between TGs and circulating endotoxin became stronger in the postprandial state each hour over the 4-h duration, substantiating previous evidence that lipids mediate the transfer of endotoxin from the gastrointestinal tract into the circulation (9,11).

Concurrent with postprandial changes in TGs, the LDL/HDL ratio reduced compared with baseline measurements. Specifically, HDL was significantly reduced at time points postprandially within all except the NOC group, potentially due to parallel elevations in chylomicrons and VLDL, as noted in other studies (34–36). Whereas it is established that obese type 2 diabetic patients suffer from a syndrome of high serum TG and low HDL (37), low levels of HDL



**Figure 2**—Increase in endotoxin levels between the NOC subjects and the obese (A), IGT (B), and type 2 diabetic (T2DM) (C) subjects from baseline to 4 h after a high-fat meal. Endotoxin is measured in EU/mL, and the percentage increase compared with NOC is also shown.

are also associated with low levels of sCD14 (soluble CD14) (38). This result corresponds with the data that endotoxin has been demonstrated to bind to HDL in the presence of sCD14 and LPS binding protein (39,40), an enzyme involved in presentation of endotoxin to sCD14. This outcome supports a role for HDL in the immunological response to endotoxin. Therefore, a reduction in HDL would reduce the removal of endotoxin further and exacerbate the inflammatory status, further compounded by higher circulating levels of endotoxin in the obese, IGT, and type 2 diabetic subject groups.

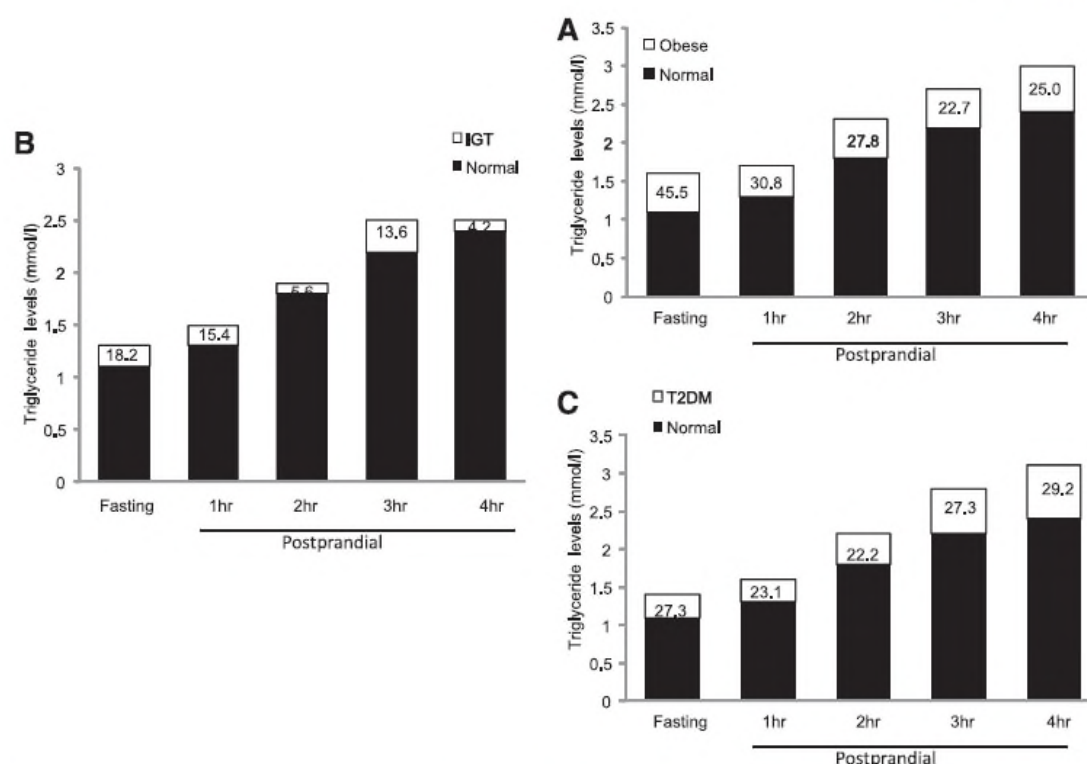
Whereas our studies have highlighted the impact of metabolic disease status on circulating endotoxin, it is important to recognize the limitations of the study. In all research, it is always preferable to increase the subject numbers that comprise each cohort. In the present studies, increased numbers might have noted different

postprandial responses to the high-fat meal within each cohort, if the groups were further subdivided. However, in light of this being a cross-sectional study, in which intra- and inter-comparisons can be made, the numbers do not detract from the findings. Consistent and significant trends were observed within the subjects over the 4-h postprandial duration, and differences between the cohorts were duly noted. We also recognize that the research subjects were given a very high-fat meal (75 g), roughly equivalent to their total daily intake of fat, which some observers might argue is an excessive (nonphysiological) amount of fat. However, despite the fat load, there was no significant change in endotoxin, cholesterol, LDL, or HDL levels postprandially in the NOC subjects in contrast to the other groups examined; the fat load administered was based on previous studies (30). Furthermore, administration of 75 g glucose could also be considered

high and would far exceed normal intake of glucose in one sitting, yet this is standard clinical practice for assessment of insulin sensitivity, whereas the fat load is only currently used as a research tool. No ill effects were noted in any of the patients during or after the study.

In summary, our current data shed new light on our understanding of metabolic endotoxemia in the postprandial state in metabolic disease. Our findings suggest that circulating endotoxin levels change depending on whether you are prediabetic, are nonobese, are obese, have IGT, or have type 2 diabetes. Further, circulating endotoxin levels noted in subjects with type 2 diabetes, at 4-h postprandial high-fat meal, far exceed our previous understanding based on other feeding studies in healthy subjects or the fasted state in type 2 diabetic subjects. Therefore, our 4-h data suggest a much higher inflammatory risk than previous





**Figure 3**—Increase in triglyceride levels between the NOC subjects and the obese (A), IGT (B), and type 2 diabetic (T2DM) (C) subjects from baseline to 4 h after a high-fat meal. Triglyceride levels are measured in mmol/L, and the percentage increase compared with NOC is also shown.

studies have indicated. These findings highlight the point that requesting patients to eat smaller, more frequent meals may actually increase their inflammatory risk further, especially in subjects with type 2 diabetes (who tend to favor high-fat foods) (32). Finally, while the most obvious solution to metabolic endotoxemia appears to be to reduce saturated fat intake, the Western diet is not conducive to this mode of action, and it is difficult for patients to comply with this request. Therefore, we need to understand the complexity of diet, meal frequency, and its acute effects on inflammatory risk and give more guidelines to particular subject groups, since leaving food intake to “individual preferences” appears not to represent a beneficial solution to reduce the inflammatory state in metabolic at-risk subjects.

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A.L.H. performed design, endotoxin experiments, and statistical analysis and drafted the manuscript. M.C.V. conducted the in vivo experiments and acquired all of the samples and anthropometric data. G.T. drafted and revised the manuscript. K.C.M. carried out the adipokine assays. N.M.A.-D. and O.S.A.-A. performed the lipid analysis. S.S. performed statistical analysis and interpretation of data. J.P.O., A.C., and P.S. provided the concept, interpreted data, and provided intellectual input. S.K. and P.G.M. provided design and concept, developed the manuscript, and performed the final revision of the manuscript. P.G.M. is also the guarantor of the article.

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# Increased Glycation and Oxidative Damage to Apolipoprotein B100 of LDL Cholesterol in Patients With Type 2 Diabetes and Effect of Metformin

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**OBJECTIVE**—The aim of this study was to investigate whether apolipoprotein B100 of LDL suffers increased damage by glycation, oxidation, and nitration in patients with type 2 diabetes, including patients receiving metformin therapy.

**RESEARCH DESIGN AND METHODS**—For this study, 32 type 2 diabetic patients and 21 healthy control subjects were recruited; 13 diabetic patients were receiving metformin therapy (median dose: 1.50 g/day). LDL was isolated from venous plasma by ultracentrifugation, delipidated, digested, and analyzed for protein glycation, oxidation, and nitration adducts by stable isotopic dilution analysis tandem mass spectrometry.

**RESULTS**—Advanced glycation end product (AGE) content of apolipoprotein B100 of LDL from type 2 diabetic patients was higher than from healthy subjects: arginine-derived AGE, 15.8 vs. 5.3 mol% ( $P < 0.001$ ); and lysine-derived AGE, 2.5 vs. 1.5 mol% ( $P < 0.05$ ). Oxidative damage, mainly methionine sulfoxide residues, was also increased: 2.5 vs. 1.1 molar equivalents ( $P < 0.001$ ). 3-Nitrotyrosine content was decreased: 0.04 vs. 0.12 mol% ( $P < 0.05$ ). In diabetic patients receiving metformin therapy, arginine-derived AGE and methionine sulfoxide were lower than in patients not receiving metformin: 19.3 vs. 8.9 mol% ( $P < 0.01$ ) and 2.9 vs. 1.9 mol% ( $P < 0.05$ ), respectively; 3-nitrotyrosine content was higher: 0.10 vs. 0.03 mol% ( $P < 0.05$ ). Fructosyl-lysine residue content correlated positively with fasting plasma glucose. Arginine-derived AGE residue contents were intercorrelated and also correlated positively with methionine sulfoxide.

**CONCLUSIONS**—Patients with type 2 diabetes had increased arginine-derived AGEs and oxidative damage in apolipoprotein B100 of LDL. This was lower in patients receiving metformin therapy, which may contribute to decreased oxidative damage, atherogenicity, and cardiovascular disease. *Diabetes* 59:1038–1045, 2010

Cardiovascular disease (CVD) is the major cause of premature death in diabetes. Type 2 diabetes is associated with a twofold to threefold increased risk of coronary heart disease in men and a threefold to fivefold increased risk in women, relative to the nondiabetic population (1). Dyslipidemia is a key feature of diabetic CVD where small dense LDL particles pose a major atherogenic threat. The underlying mechanism producing small, dense LDL is related to hepatic oversecretion of apolipoprotein B100 (apoB100) and impaired clearance of LDL by the high-affinity LDL receptor in which both hepatic and peripheral tissues participate (2). The normal residence time of LDL in plasma is 3 days but this is increased to 5 days for small, dense, highly atherogenic LDL (3). Atherogenicity and plasma residence time of LDL may be influenced by damage to apoB100 by glycation, oxidation, and nitration but the quantitative amounts of damage in healthy human subjects and diabetic patients remain unclear.

Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. Early stage reactions are directed to lysine and  $\text{NH}_2$ -terminal amino acid residues leading to the formation of the early glycation adduct, fructosyl-lysine (FL), and other fructosamine derivatives. Later stage reactions form advanced glycation end products (AGEs). FL degrades slowly to form AGEs. Glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG) are physiological dicarbonyl metabolites and potent glycation agents formed by the degradation of glycolytic intermediates, glycated proteins, and lipid peroxidation. They react with proteins to form AGEs directed mainly to arginine residues—often functionally important arginine residues. The most important AGEs quantitatively are hydroimidazolones derived from arginine residues modified by glyoxal, methylglyoxal, and 3-DG: G-H1, MG-H1, and 3DG-H, respectively.  $\text{N}_\omega$ -carboxymethyl-arginine (CMA) is a further arginine-derived adduct formed by glyoxal. Other important and widely studied AGEs are  $\text{N}_\epsilon$ -carboxymethyl-lysine (CML),  $\text{N}_\epsilon$ -carboxyethyl-lysine (CEL), and pentosidine. Markers of oxidative damage to proteins are methionine sulfoxide (MetSO), formed by the oxidation of methionine, and dityrosine, formed by oxidative cross-linking of tyrosine. A widely studied marker of nitration damage to proteins is 3-nitrotyrosine (3-NT) (rev. in 4) (Fig. 1).

Metformin is the most widely prescribed oral glucose-lowering agent for the treatment of type 2 diabetes. It improves glycemic control and decreases the risk of CVD (5). Metformin therapy of type 2 diabetic patients increased LDL particle size (6) and decreased plasma con-

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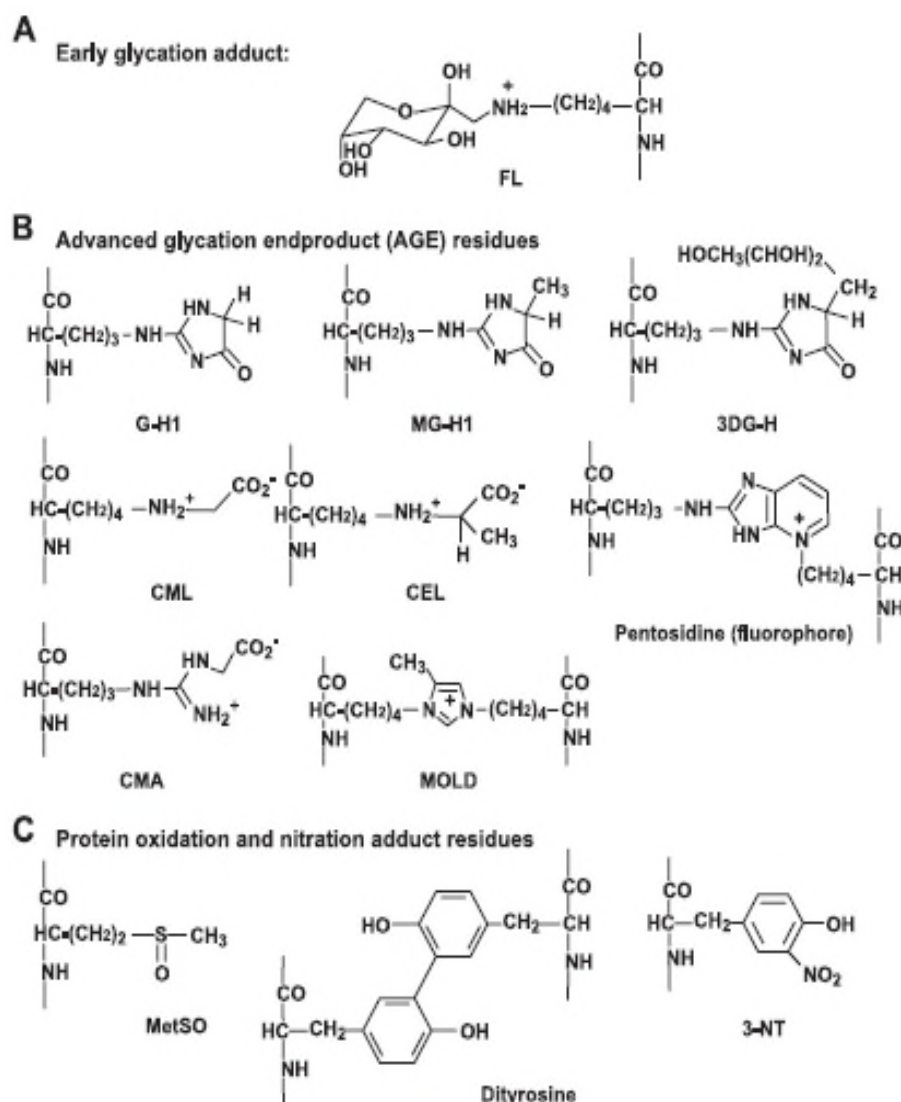


FIG. 1. Molecular structures of protein glycation, oxidation, and nitration residues.

centrations of remnant lipoprotein cholesterol, a predictor of myocardial infarction and thought to reflect increased residence time and atherogenicity of cholesterol ester-rich chylomicrons and VLDL (7). Metformin also decreased the plasma concentrations of methylglyoxal in diabetic patients (8) and may decrease oxidative stress and related oxidation of LDL (9).

In this study, we used the gold standard method of stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure protein glycation, oxidation, and nitration adducts in apoB100 of LDL to assess whether there is increased lipoprotein damage in patients with type 2 diabetes with respect to normal healthy control subjects and to investigate the effect of metformin therapy.

#### RESEARCH DESIGN AND METHODS

**Patients and normal healthy volunteers.** Diabetic patients were recruited from patients attending the Diabetes Clinics at Colchester General Hospital (Colchester, U.K.) and University Hospital of Coventry and Warwickshire

(Coventry, U.K.). Healthy control volunteers were recruited from partners and friends of the patients and investigators. Ethical approval for the study was given by the local ethics committees (North and Mid-Essex Local Research Ethics Committee, Chelmsford, U.K. and Coventry Research Ethics Committee, Coventry, U.K.). Inclusion criteria were type 2 diabetes with normoalbuminuria (albumin excretion rate <30 mg/24 h), age 40–80 years, diabetes duration of  $\geq 1$  year, and A1C <13%. Exclusion criteria were individuals with significant comorbidities who participated in an intervention study within 90 days, recipients of renal and/or pancreatic transplants, and women who were pregnant or breastfeeding or of child-bearing potential not using adequate contraceptive precautions. Metformin therapy was given in the dose range 0.85–3 g/day; median 1.50. The duration of metformin therapy was in the range 1–20 years; median 4 years. Other therapy (number of patients without/with metformin therapy) was insulin (17/5), glimepiride (2/3), glimepiride (1/2), and antihypertensive therapy (0/4). Participant characteristics are shown in Table 1. Venous blood samples (fasting) were taken after informed consent. Plasma was separated immediately and stored at  $-80^{\circ}\text{C}$  until analysis.

**Isolation of LDL.** For rapid, same-day preparation of LDL, a self-generating gradient of iodixanol in a vertical rotor (S120VT) was used in a Sorvall MTX 160 microultracentrifuge (Hitachi). The density of plasma was increased to 1.25 using 60% iodixanol solution (OptiPrep; Axis-Shield). Plasma (0.9 ml) was layered under 0.9 ml of 0% iodixanol in a 2-ml ultracentrifuge tube (polyal-



TABLE 1  
Characteristics of type 2 diabetic patients and normal healthy control subjects

Study group	Control subjects	All type 2 diabetic subjects	Type 2 diabetic subjects not receiving metformin therapy	Type 2 diabetic subjects receiving metformin therapy
<i>n</i>	21	32	19	13
Age (years)	56.5 ± 0.7	60.5 ± 12.2	64.1 ± 12.8*	55.2 ± 0.5†
Sex (M/F)	10/11	14/18	11/8	3/10
BMI (kg/m <sup>2</sup> )	28.6 ± 3.6	33.7 ± 6.3‡	31.5 ± 6.3	37.1 ± 4.8†‡
Duration of diabetes (years)	—	11 (1–35)	13 (1–35)	8 (1–25)
FPG (mM)	5.14 ± 0.74	8.75 ± 2.51‡	9.15 ± 2.41‡	8.17 ± 2.62§
HbA <sub>1c</sub> (%)	5.48 ± 0.57	8.20 ± 1.94	8.25 ± 2.04‡	8.13 ± 1.85‡
Total cholesterol (mM)	5.11 ± 1.43	4.90 ± 1.02	4.71 ± 1.00	5.18 ± 1.01
LDL cholesterol (mM)	3.18 ± 1.41	3.01 ± 1.01	2.89 ± 1.04	3.17 ± 0.98
HDL cholesterol (mM)	1.55 ± 0.81	1.24 ± 0.30	1.18 ± 0.23	1.34 ± 0.36
Triglycerides (mM)	1.16 ± 0.31	2.23 ± 1.02‡	2.13 ± 1.09§	2.37 ± 0.94‡
Systolic blood pressure (mmHg)	130 ± 20	141 ± 22	140 ± 24	142 ± 21
Diastolic blood pressure (mmHg)	77 ± 8	78 ± 8	77 ± 10	78 ± 10
GFR (ml/min)	99 ± 20	100 ± 42	89 ± 30	116 ± 52

Data are mean ± SD or median (minimum – maximum). Significance: \* $P < 0.05$ , † $P < 0.01$ , and ‡ $P < 0.001$ , with respect to normal healthy control subjects; and § $P < 0.05$ , with respect to type 2 diabetic patients receiving conventional therapy. GFR, glomerular filtration rate.

lomer, no. S302807A; Hitachi) and further void filled with 0.2 ml nitrogen-purged PBS. The sample was centrifuged (101,000g, 16°C, 2.5 h) with low acceleration and deceleration. LDL was washed with nitrogen-purged water (4 ml × 3) over 100-kDa microspin filters (Amicon) to remove iodixanol. The LDL was stored at –20°C until further analysis. Sample handling was performed under subdued light. Protein concentration was measured by Bradford assay. Lipid peroxidation of LDL was assessed by measuring thiobarbituric acid reactive substances (TBARS). TBARS were quantified by reference of the chromophoric response to a standard curve constructed from malondialdehyde tetramethyl acetal and expressed as malondialdehyde equivalents (10). The purity of LDL was assessed by SDS-PAGE denaturing and agarose nondenaturing electrophoresis.

**In vitro modification of LDL.** LDL was glycated minimally by methylglyoxal and glucose *in vitro* to assess the major glycation adducts formed. LDL glycated minimally by methylglyoxal (MG<sub>min</sub>-LDL) was prepared by incubation of methylglyoxal (200 μmol/l) with LDL (4.2 mg/ml) in PBS (0.4 mmol/l diethylenetriaminepentaacetic acid [DETAPAC], pH 7.4) at 37°C for 6 h. The glycated and control LDL was washed extensively with argon-purged ice-cold water using ultra-spin filters (Amicon 100-kDa cutoff membrane from Millipore) at 4°C, and stored at 4°C under argon and used within 2 weeks. LDL glycated minimally by glucose (AGE<sub>min</sub>-LDL) was prepared by incubation of glucose (25 mmol/l) with LDL (3 mg/ml) in PBS (0.4 mmol/l DETAPAC, pH 7.4) under argon (0.5 ml) at 37°C for 7 days under sterile conditions. Control LDL was incubated without glucose. The glycated and control LDL was washed with argon-purged water ultrafiltration at 4°C, and stored at 4°C under argon until further analysis. Electrophoretic mobility of native and modified LDL on agarose gel electrophoresis was performed using a gel lipoprotein electrophoresis kit using barbital buffer, pH 8.6 (Hoefer).

**Delipidation of LDL.** An aliquot of LDL solution (20 μl, 100 μg) was transferred into a glass tube (50 × 7.5 mm) containing butylated hydroxytoluene in methanol (5 μl, 2 mg/ml), 20% trichloroacetic acid (100 μl), and water (75 μl), mixed well, left on ice for 10 min, and then centrifuged (10,000g, 15 min, 4°C). The supernatant was removed and the pellet washed with acetone (200 μl) and diethyl ether (200 μl) and dried under argon.

**Enzymatic digestion of apoB100.** Delipidated protein was hydrolyzed exhaustively by modification of our published procedure (11). Protein was suspended in 100 mmol/l potassium phosphate buffer, pH 7.4 (50 μl). Pronase E (20 μl, 2 mg/ml in 10 mmol/l KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) and 10 μl penicillin (50 units/ml) and streptomycin (50 μg/ml) were added and the samples incubated at 37°C for 24 h. Thereafter, 10 μl each of prolidase and aminopeptidase solutions (2 mg/ml in 10 mmol/l KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4) was added, and samples were incubated for a further 48 h. All steps were performed under argon. A similar method was used previously to quantify the oxidative marker 6-hydroxy-2-aminovaleric acid in apoB100 (12).

**Protein biomarker determination by LC-MS/MS.** Fructosyl-lysine, advanced glycation end products, and oxidation and nitration markers were determined in enzymatic hydrolysates of delipidated lipoprotein by stable isotopic dilution analysis LC-MS/MS (13).

**Statistics.** Data are mean ± SD for parametric data and median (minimum – maximum) or (lower – upper quartile) for nonparametric data. Significance of

difference between means was assessed by Student *t* test and significance of difference between medians, by the Mann-Whitney *U* test. Difference of proportions was assessed using Fisher contingency tables.

## RESULTS

**Isolation of LDL and glycation by methylglyoxal and glucose *in vitro*.** A new rapid method for isolation of LDL was developed and used in this study. This method used a single ultracentrifugation step for 2.5 h only with samples at 16°C (14). High purity was confirmed by a single protein band in denaturing SDS-PAGE and agarose nondenaturing electrophoresis (data not shown). Lipid peroxidation of isolated LDL from normal healthy control subjects, as judged by TBARS content, was low: 0.81 ± 0.45 nmol/mg protein ( $n = 12$ ).

LDL glycated minimally by methylglyoxal and glucose showed increased levels of AGE residues. For glycation by methylglyoxal, MG<sub>min</sub>-LDL showed increased content of MG-H1, CEL, and MOLD residues. The major AGE formed by glycation with methylglyoxal was MG-H1 (98.4%) with minor formation of CEL (1.4%) and MOLD (0.2%). For glycation of LDL by glucose, the major increase in glycation adducts was of FL residues with a minor increase in CML residues (Table 2).

**Protein damage markers in apolipoprotein B100 of LDL of healthy human subjects and patients with type 2 diabetes.** In apoB100 of LDL of healthy human subjects, the mean FL residue content was 2,900 pmol/mg apoB100, equivalent to 1.49 mol/mol apoB100 or 4.17 mmol/mol Lys. Major AGE residues quantitatively were MG-H1, median content 46.8 pmol/mg apoB100, equivalent to 0.024 mol/mol apoB100 or 0.16 mmol/mol Arg; and CML, median content 24.0 pmol/mg apoB100, equivalent to 0.012 mol/mol apoB100 or 0.035 mmol/mol Lys. Median total arginine-derived AGE residue content (G-H1 + MG-H1 + 3DG-H + CMA + pentosidine) was 103 pmol/mg apoB100, equivalent to 0.062 mol/mol or 0.42 mmol/mol Arg. Median total lysine-derived AGE residue content (CML + CEL + MOLD + pentosidine) was 33 pmol/mg apoB100, equivalent to 0.017 mol/mol or 0.047 mmol/mol Lys. The major oxidative marker was MetSO residues with mean content of 2084 pmol/mg apoB100, equivalent to 1.07 mol/mol apoB100 or



TABLE 2  
Changes in protein glycation adduct residues in human LDL minimally modified by methylglyoxal and glucose in vitro

Glycation adduct	Control 1	MG <sub>min</sub> -LDL	Control 2	AGE <sub>min</sub> -LDL
FL	2.21 ± 0.21	1.99 ± 0.06	3.10 ± 0.67	6.08 ± 0.37*
CML	0.031 ± 0.004	0.032 ± 0.007	0.056 ± 0.009	0.070 ± 0.003†
CEL	0.004 ± 0.001	0.024 ± 0.003*	0.011 ± 0.003	0.013 ± 0.003
MG-H1	0.15 ± 0.02	1.57 ± 0.37*	0.23 ± 0.03	0.21 ± 0.01
MOLD	0.0002 ± 0.0001	0.0025 ± 0.0009*	0.0057 ± 0.001	0.0092 ± 0.002

Data are mol adduct/mol apoB100; mean ± SD (n = 3). Control 1 and control 2 are incubations of LDL for 6 h and 7 days without methylglyoxal and glucose, respectively. Significance: \*P < 0.001, †P < 0.01. Other adduct residues, G-H1, 3DG-H, CMA, pentosidine, MetSO, dityrosine, and 3-NT, were not changed significantly during the incubation with glycation agents.

13.7 nmol/mol Met. The nitration marker 3-NT had a median residue content of 2.3 pmol/mg apoB100, equivalent to 0.0012 mol/mol apoB100 or 0.0078 nmol/mol Tyr (Table 3).

Considering all type 2 diabetic patients studied, the mean fasting plasma glucose (FPG) concentration was increased 70% and glycated hemoglobin was increased by 2.7% total hemoglobin with respect to control subjects (Table 1). FL residue content of apoB100 of LDL was not increased significantly. Marked increases were found, however, for contents of dicarbonyl-derived AGE residues: CEL, fivefold, G-H1 ninefold, MG-H1 fourfold, 3DG-H threefold, MOLD fivefold, and pentosidine threefold. Median total arginine-derived AGE residue content was increased more than threefold, 316 vs. 103 pmol/mg apoB100 (P < 0.001); whereas total lysine-derived AGE residue content was increased only 47%, 49 vs. 33 pmol/mg apoB100 (P < 0.05). For oxidative markers, MetSO residue content of apoB100 of type 2 diabetic patients was increased twofold and dityrosine residue content was increased 64-fold. 3-NT residue content of apoB100 was decreased 61% in type 2 diabetic patients (Table 3). Only one protein damage marker of apoB100 in diabetic patients was linked to donor sex: median CML content was 13.3 pmol/mg for males and 30.3 for females (P < 0.05).

**Correlation analysis for markers of glycemic control and protein damage in plasma apoB100 of type 2 diabetic patients.** There was no correlation of protein damage marker content of apoB100 with patient age,

suggesting that the significant age difference of diabetic patients with and without metformin therapy did not compromise protein damage marker of these study groups. For markers of glycemic control, FPG concentration correlated positively with A1C and also with FL residue content of apoB100. FL residue content correlated positively with CEL, MG-H1, and 3DG-H residue contents. There was a cluster of correlations of dicarbonyl-derived AGE residue contents: G-H1 correlated positively with MG-H1 and CMA; MG-H1 also correlated positively with CMA and 3DG-H, and also with CML and pentosidine; and 3DG-H correlated positively with CMA. There was also a cluster of correlations of oxidative marker residues with AGE residue contents: MetSO correlated positively with CEL, G-H1, MG-H1, 3DG-H, CMA, and pentosidine; and CML and MOLD correlated positively with dityrosine (Table 4). There were negative correlations of 3-NT with MG-H1 and 3DG-H.

**Protein damage markers in apolipoprotein B100 of LDL of patients with type 2 diabetes receiving metformin.** Patients receiving metformin therapy were slightly younger and more obese than those not receiving metformin therapy, although all other conventional clinical variables were not significantly different (Table 1). ApoB100 of LDL from patients receiving metformin therapy had lower contents of AGEs (G-H1, MG-H1, 3DG-H, and CMA) and MetSO but higher 3-NT content

TABLE 3  
Markers of protein damage in apolipoprotein B100 of LDL

Type of modification/ Analyte	Control subjects	All type 2 diabetic subjects	Type 2 diabetic subjects not receiving metformin therapy	Type 2 diabetic subjects receiving metformin therapy
n	21	32	19	13
Fructosamine				
FL	2,900 ± 1,402	3,347 ± 1,914	3,789 ± 1,971	2,682 ± 1,688
AGE				
CML	24.0 (0.7–143.7)	20.6 (1.7–58.9)	20.5 (3.9–58.9)	24.1 (1.7–56.4)
CEL	3.5 (0.2–38.9)	17.3 (3.5–59.6)*	21.9 (3.5–59.6)*	14.6 (4.6–33.4)†
G-H1	3.6 (0.1–50.4)	31.5 (1.2–188.3)	44.0 (1.8–188.3)*	25.0 (1.2–59.0)†‡
MG-H1	46.8 (15.9–219.4)	197.0 (3.0–474.4)*	235.8 (45.5–474.4)*	91.3 (3.0–309.4)§
3DG-H	19.4 (2.2–138.9)	60.0 (4.8–163.8)*	82.3 (4.8–163.8)*	39.4 (6.3–86.2)‡
CMA	20.3 (0.4–47.9)	26.8 (0.7–112.8)	38.3 (0.7–112.8)†	8.9 (1.6–74.6)‡
MOLD	1.8 (0.3–51.5)	9.0 (0.2–31.8)‡	12.2 (0.2–31.8)‡	7.6 (1.5–27.3)†
Pentosidine	0.26 (0.03–0.84)	0.76 (0.08–2.13)*	0.75 (0.18–2.13)*	0.76 (0.08–1.61)‡
Oxidation				
MetSO	2,084 ± 1,360	4,738 ± 3,367*	5,633 ± 3,837*	3,857 ± 2,641‡
Dityrosine	0.26 (0.05–6.86)	16.7 (0.2–68.4)*	16.8 (5.8–34.8)*	11.0 (0.2–47.1)*
Nitration				
3-NT	2.3 (0.3–49.1)	0.9 (0.1–24.1)†	0.7 (0.1–15.4)‡	2.0 (0.1–24.1)‡

Data are pmol/mg apoB100; mean ± SD or median (minimum – maximum). Significance: †P < 0.05, ‡P < 0.01, and \*P < 0.001, with respect to normal healthy control subjects; and ‡P < 0.05 and §P < 0.01, with respect to type 2 diabetic patients not receiving metformin therapy.



Glycemic control		AGE											Oxidative damage		
Analyte	HbA <sub>1c</sub>	FL	CML	CEL	G-H1	MG-H1	3DG-H	CMA	MOLD	Pent	MetSO	DT	3-NT		
	FPG	0.46**													
	HbA <sub>1c</sub>	0.58***													
AGE	CML														
	CEL		0.39*												
	G-H1														
	MG-H1		0.42*	0.42*		0.67***									
	3DG-H		0.43*				0.76***								
	CMA				0.61***		0.80***	0.68***							
	MOLD			0.51**											
	Pent			0.37*			0.41*								
Oxidative damage	MetSO				0.38*	0.60***	0.62***	0.43*	0.77***		0.53**				
	DT			0.63***						0.68**					
	3-NT						-0.31*	-0.55**							
	Analyte	FPG	HbA <sub>1c</sub>	FL	CML	CEL	G-H1	MG-H1	3DG-H	CMA	MOLD	Pent	MetSO		
		Glycemic control						AGE					Oxidative damage		

than apoB100 of LDL from patients not receiving metformin therapy. MG-H1, CMA, MetSO, and 3-NT residue contents of apoB100 of LDL from diabetic patients receiving metformin therapy were not significantly different from those of normal healthy subjects (Fig. 2 and Table 3).

A new method for rapid isolation of LDL is described and used here with a single ultracentrifugation step of only 2.5 h at 16°C, whereas the conventional method of LDL isolation involves ultracentrifugation for 20–22 h at 15°C (14). This rapid method has potential to decrease the risk of apoB100 damage in preanalytic processing and could facilitate clinical studies of LDL.

apoB100 of healthy control was ~56. ApoB100 is probably also glycated prior to assimilation into LDL.

The protein damage marker of highest quantitative content in apoB100 of LDL in healthy human subjects was the early glycation adduct FL, equivalent to 0.42% lysine residues. These levels are threefold lower than reported in earlier studies using the tritiated borohydride reduction technique (1.3% [16]) but similar to the 2–3 nmol FL residues per milligram apoB100 estimates using the furfural technique (17). FL, MG-H1, MetSO, and 3-NT are major adducts of early glycation, advanced glycation, oxidation, and nitration of apoB100, LDL, and also total plasma protein. The rates of damage of LDL and plasma protein can be predicted, assuming these rates are equal to the rate of clearance of adducts in the steady state and taking into account half-lives of LDL and serum albumin—the major plasma protein—are ~3 and 19 days, respectively (18,19). The outcome of these predictions is shown in Table 5. Estimates of  $k_{\text{LDL, Glucose}}$  [ $69 \text{ (mol/l)}^{-1} \cdot \text{day}^{-1}$ ] and  $k_{\text{LDL, MG}}$  [ $55,452 \text{ (mol/l)}^{-1} \cdot \text{day}^{-1}$ ] from these deductions were not markedly dissimilar from estimates from in vitro glycation studies (see above). Overestimation of rates from in vivo data may be attributed to glycation of apoB100 prior to assimilation in LDL particles. Overall apoB100 of LDL is far more reactive to damage by these modifications than is albumin, even when the eightfold greater molecular mass of apoB100 relative to albumin is taken into account. ApoB100 is highly susceptible to damage and may be a particularly good sensor of it. From the predicted in situ rates of modification, the rate of early and advanced glycation of LDL is only 17 and 4% of that of albumin, whereas the in situ rates of oxidation and nitration are 10 and 70% greater than those of albumin. As LDL has a short plasma half-life, however, the steady levels of protein glycation, oxidation, and nitration adducts in apoB100 represent only a minor part of the total plasma adduct concentration (Table 5). The adduct content in apoB100 of diabetic patients may be increased by effects of both increased rate of modification, caused by increased plasma concentrations of modifying agents, and decreased rate of elimination.

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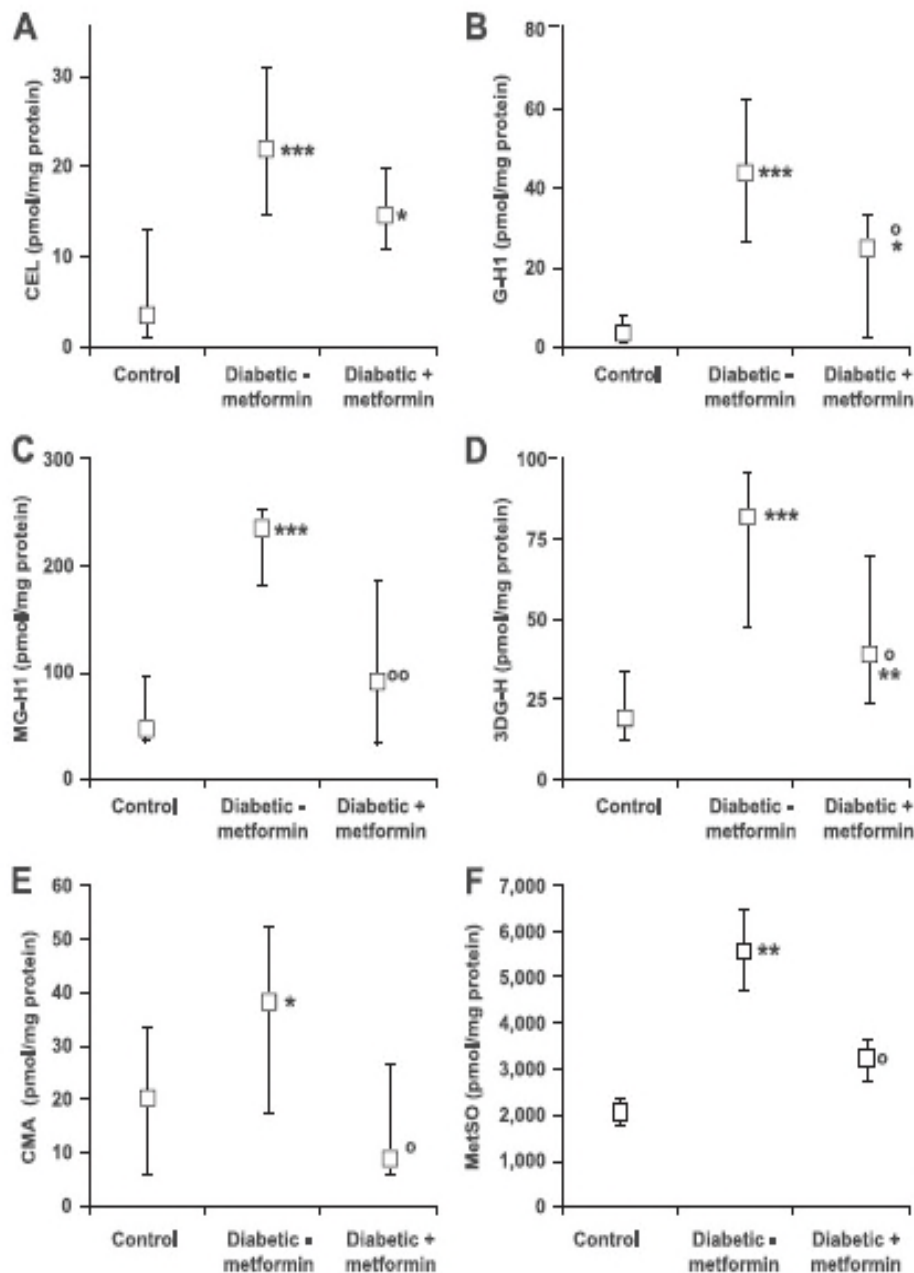


FIG. 2. Advanced glycation end product and methionine sulfoxide residue contents of apolipoprotein B100 of LDL of type 2 diabetic patients with and without metformin therapy. (A) CEL, (B) G-H1, (C) MG-H1, (D) 3DG-H, (E) CMA, and (F) MetSO. Data are median (lower – upper quartile) except for MetSO, which is mean  $\pm$  SD. Significance: \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , with respect to normal healthy control subjects; and o $P < 0.05$  and oo $P < 0.01$ , with respect to type 2 diabetic patients not receiving metformin therapy.

content of apoB100 is not significantly different from that of apoB100 from healthy subjects. Increased plasma glucose concentration in type 2 diabetic patients did not produce a significant increase in FL residue content of apoB100. This may indicate that formation of FL residues in apoB100 by glucose is less favored when the extent of glycation exceeds 2 molar equivalents, limiting further increase of FL residue content in diabetes. Dicarbonyl-derived AGE content of apoB100 from type 2 diabetic patients was, however, increased markedly. This suggests that dicarbonyl glycation is the main cause of increased AGE content of apoB100 of LDL in type 2 diabetic patients.

Arginine-derived AGE residue contents of apoB100 in these patients increased more than threefold.

Major quantitative oxidative damage—MetSO residue content—of apoB100 in type 2 diabetic patients was twofold higher than in apoB100 of healthy control subjects. This is commensurate with increased plasma peroxide concentration in type 2 diabetes (21). There is no repair of MetSO by MetSO reductase in plasma, hence plasma MetSO likely reflects increased plasma reactive oxygen species in diabetes and decreased plasma reactive oxygen species production for patients treated with metformin. The 64-fold increase of dityrosine residue content



TABLE 5

Comparison of the predicted reactivity of apolipoprotein B100 of LDL and total plasma protein toward early glycation, advanced glycation, oxidation, and nitration

Type of modification	Adduct	ApoB100 of LDL content (nmol/mol amino acid modified)	Plasma protein content* (nmol/mol amino acid modified)	$k_{LDL}/k_{Albumin}$	$r_{LDL}/r_{Albumin}$	LDL adduct content ( $\mu M$ )	Total plasma protein adduct content ( $\mu M$ )
Early glycation	FL	4.17	1.35	93	0.17	1.9	71.8
Advanced glycation	MG-H1	0.16	0.31	20	0.038	0.031	5.20
Oxidation	MetSO	13.7	1.93	503	1.10	1.4	9.2
Nitration	3-NT	0.0078	0.0006	943	1.76	0.0015	0.0055

$k_{LDL}/k_{Albumin}$  is the predicted ratio of the rate constants for modification of apoB100 of LDL and albumin, and  $r_{LDL}/r_{Albumin}$  is the predicted ratio of in situ rates of modification of apoB100 of LDL and albumin (the latter taking into account the concentrations of LDL and albumin in plasma) by glucose to form FL, methylglyoxal to form MG-H1, oxidation to form MetSO, and nitration to form 3-NT. Assumptions: the rate of formation of FL, MG-H1, MetSO, and 3-NT in LDL and plasma protein is equal to the rate of clearance of protein adducts; half-lives of LDL and human serum albumin are ~3 and 19 days, respectively (18, 19); plasma concentrations of apoB100 of LDL and albumin are 1.28  $\mu mol/l$  (equivalent to 3.18 nmol/l LDL cholesterol) and 682  $\mu mol/l$  (equivalent to 44 mg/ml), respectively, total plasma protein concentration 84 mg/ml, and plasma protein amino acid contents: Lys 820 nmol/mg, Arg 262 nmol/mg, Met 164 nmol/mg, and Tyr 183 nmol/mg protein (13). All protein damage in plasma protein other than that of apoB100 of LDL is attributed to adducts of albumin. \*Data are from ref. 20.

of apoB100 in diabetic patients, however, far exceeds this. Dityrosine residues are formed by both spontaneous and enzymatic processes. Enzymatic formation is catalyzed by dual oxidase-1 (22)—a member of the NADPH oxidase family of enzymes implicated in signaling in vascular disease in diabetes (23). Activation of NADPH oxidase/dual oxidase isozymes in diabetes may markedly enhance the formation of dityrosine residues in apoB100 (24). Dityrosine content of apoB100 of diabetic patients (~0.06 nmol/mol Tyr) was intermediate between that of normal control subjects (~0.001 nmol/mol Tyr) and of apoB100 isolated from atherosclerotic plaques (~0.25 nmol/mol Tyr) (25).

For type 2 diabetic patients receiving metformin therapy, there were lower contents of dicarbonyl-derived AGE and MetSO residues than in patients not receiving metformin therapy. CML residue content of apoB100 of LDL was linked to patient sex; imperfect matching of sex may have masked change in CML residue content in patients receiving metformin therapy. Metformin decreased the concentration of methylglyoxal in type 2 diabetic patients (8). It is also expected to react with glyoxal and 3-DG similarly, and thereby decrease plasma levels of these dicarbonyls and prevent related formation of AGE residues. Metformin reacts with methylglyoxal in vivo, forming a triazepinone adduct that has been detected in plasma and urine (26). This decreases methylglyoxal by a scavenging action, although the relatively slow kinetics of this reaction prompted consideration of other mechanisms (27). Improvement of glycaemic control by metformin decreases dicarbonyl formation and thereby decreased AGE formation of apoB100 indirectly, as suggested by the correlation of FL residue content of apoB100 with contents of CEL, MG-H1, and 3DG-H residues. Both mechanisms are likely involved.

A remarkable finding was the decrease in MetSO residue content of apoB100 in patients receiving metformin therapy. In correlation analysis, there were strong correlations of MetSO with G-H1, MG-H1, and CMA residue contents of apoB100. These correlations were not found in similar analysis of total plasma protein (28), which suggests these relationships are specific to LDL. This likely relates to the most important physiological impact of our findings: our recent research suggests that formation of MG-H1 residues in apoB100 increases binding of LDL to proteoglycan, which may increase the half-life of LDL in the extracellular

compartment and thereby susceptibility to oxidation (29). Decreased fractional clearance of apoB100 has been linked to oxidative damage of apoB100 and atherogenicity (12). Increased binding to proteoglycan in the subendothelium is thought to be integral to this process (30). Metformin may decrease dicarbonyl glycation of apoB100 and in so doing prevent decreased plasma clearance and increased oxidation and atherogenicity of LDL in type 2 diabetes. In future studies, it will be of interest to test this hypothesis in prospective placebo-controlled studies.

In this study, 3-NT residue content of apoB100 of LDL was lower in diabetic patients not receiving metformin therapy than healthy control subjects and normalized in patients with metformin therapy. The quantitative amount of 3-NT residues (0.03–0.1 mol%) is unlikely to be damaging, but it may be a marker of nitric oxide bioavailability. Metformin therapy has recently been shown to be linked to activation of endothelial nitric oxide synthase (31). The changes in 3-NT residues of apoB100 of LDL here may reflect bioavailability of nitric oxide in diabetic patients, thereby suggesting that diabetic patients receiving metformin therapy may achieve normal vascular nitric oxide bioavailability. This provides a further mechanism how metformin may be protective to the vasculature in diabetes.

#### ACKNOWLEDGMENTS

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# Microvascular and cardiovascular disease in South Asians: the emerging challenge

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## Introduction

Type 2 diabetes mellitus is the denominator for both microvascular and cardiovascular disease (CVD). Currently, 20% of all cases of type 2 diabetes in the UK are represented by people of Asian extraction and the numbers continue to rise.<sup>1</sup> Over two million South Asian people reside in the UK representing 4% of the total population.<sup>1</sup> Patients of South Asian ethnic background (UK decennial census categories [Indian, Pakistani and Bangladeshi]) with type 2 diabetes present special management problems. Diabetes is around four times more common, onset may be over a decade earlier, and there is a higher risk of complications, particularly cardiovascular and renal but also retinopathy. There is increased morbidity and a substantial increase in mortality compared to the local Caucasian population.<sup>2,3</sup> Cultural and communication differences make health care delivery more challenging in these patients and may further complicate the achievement of defined targets.<sup>4</sup>

## Type 2 diabetes and CVD in the South Asian population

Type 2 diabetes and its associated complications are becoming an emerging epidemic for the health care of people in the UK and globally. South Asians have a six-fold increase in their risk of developing type 2 diabetes compared to age matched Caucasian populations.<sup>2,3,5-8</sup> South Asians present with diabetes at a younger age; they are therefore exposed to diabetes for a longer

## ABSTRACT

South Asians now constitute 4% of the UK population and represent a significant growing minority ethnic group with distinct health care needs. Type 2 diabetes mellitus is already endemic and growing exponentially in this ethnic group. This presents a unique challenge to the overstretched resources of the NHS but, if action is not taken now, it is likely to prove highly expensive in the medium to long term. Type 2 diabetes is never mild and the microvascular and cardiovascular complications of diabetes appear to be more aggressive in the South Asians and threaten to match the exponential rise of the disease in them. Primary prevention through lifestyle changes to reduce obesity and improve diet with the aggressive reduction through pharmacological and non-pharmacological treatments of the reversible risk factors remains the cornerstone of a systematic strategy.

In this review, we will describe the problem of microvascular and cardiovascular disease in South Asians and the known causes. Finally, we will describe the United Kingdom Asian Diabetes Study, a novel culturally centred approach using a community-based health care team in an attempt to improve microvascular and cardiovascular risk in South Asians. Copyright © 2007 John Wiley & Sons.

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## KEY WORDS

South Asian; type 2 diabetes; microvascular and cardiovascular risk; central/abdominal obesity; United Kingdom Asian Diabetes Study (UKADS)

period than Caucasians, and this in turn leads to a greater risk of cardiovascular and microvascular complications. In addition to this, many surveys have shown that as many as 40% of people among UK South Asian communities have undiagnosed diabetes.<sup>8</sup> By the time they are diagnosed, many of these patients will present with significantly advanced cardiovascular and microvascular complications.<sup>9</sup> Indeed, it is because of these complications that many of these patients are found to have diabetes.

Ischaemic heart disease remains the major cause of death in the UK. South Asian men and women have a 50% higher mortality compared to Caucasian controls.<sup>2,3</sup> (Figure 1.) This risk for heart disease is seen in all UK South Asian groups and appears to be as strong in the second

generation<sup>10</sup> as in their parents. South Asians also appear to be presenting with infarction up to five years earlier.<sup>11</sup> South Asian women are not immune from this higher cardiovascular risk<sup>12</sup> and this is often underestimated by the health care profession whilst assessing them.

The difference in the death rates between South Asians and the rest of the population is widening. The death rate from coronary heart disease is not falling as fast in South Asians as it is in the rest of the population.<sup>13,14</sup>

## Why are South Asians at high risk of CVD?

This remains a key question which needs more research to be fully explained. Within the South Asian population the conventional risk fac-

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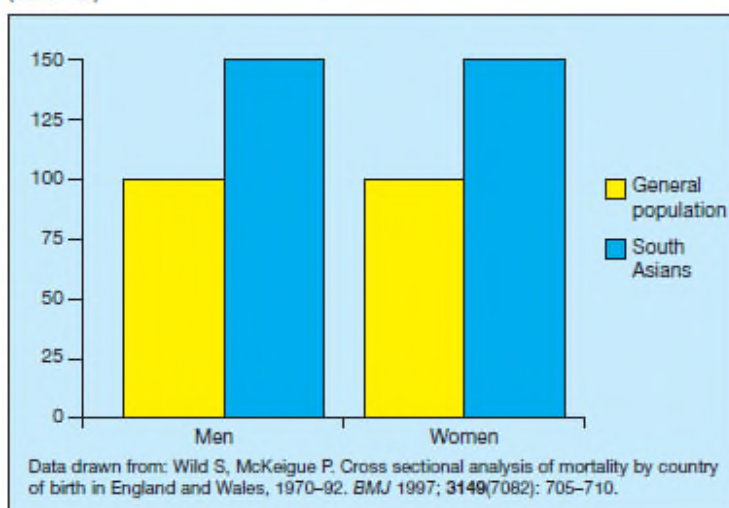
tors predict those who will go on to develop heart disease and, within the distinct ethnic subgroups of South Asians, major cardiovascular risk factors can vary considerably. For example, smoking is much less common in Sikhs than in Hindus but is much higher in Bangladeshis.<sup>13</sup> Dietary habits are also relatively diverse and unlikely to provide a simple uniform explanation.

Research studies to date have not clearly established whether prevalence rates for hypertension are higher in South Asians,<sup>4,6,7</sup> and certainly in the Bangladeshi subgroup appear to be lower.<sup>13</sup> While the increased cardiovascular mortality cannot be explained by a difference in hypertension, it has been postulated that South Asians may be more prone to the cardiovascular and microvascular complications of raised blood pressure (BP) because of their diabetes and insulin resistance.<sup>6</sup> Also, conventional definitions of high BP may not be applicable to this high risk group.<sup>15</sup>

The next important conventional risk factor to consider is unfavourable lipid profile. The pattern of lipid profile differs between South Asians compared to white Caucasians but total cholesterol is certainly not greater.<sup>16</sup> Raised triglycerides and a slightly reduced HDL-cholesterol,<sup>6,13</sup> patterns characteristic of the metabolic syndrome, have been reported. More recently, metabolically active adipose tissue is rapidly gaining evidence and prominence as one of the most important factors involved with insulin resistance and related complications. Reduced levels of adiponectin, a hormone from fat cells which is related to insulin resistance, have been reported in South Asians<sup>17</sup> supporting the emerging hypothesis that changes in adipocyte distribution and function play a role in enhancing cardiovascular risk.<sup>18</sup>

Finally, reduced physical activity with a modernised sedentary lifestyle<sup>19</sup> coupled with an increased genetic susceptibility to deposit intra-abdominal fat may lead to metabolic changes that enhance cardiovascular risk.<sup>6,18</sup> Increased insulin resistance is more common in South Asians than in Caucasians<sup>6,18</sup> and may be the key link between the pathogenesis of diabetes

**Figure 1.** Standardised chronic heart disease mortality ratios in UK South Asians compared to the general population, for individuals aged 20–69 years (1989–92)



and CVD. Insulin resistance is linked to central/abdominal obesity. The body mass index (BMI) of UK South Asians is not higher than Caucasians but there is proportionally more abdominal obesity and the deposition of more intra-abdominal fat, which is metabolically active, is strongly related to insulin resistance.<sup>6,20</sup> BMI may underestimate 'adiposity' in South Asians and, in its effect on cardiovascular risk, a value of 27.5 in this group compares to a BMI value of 30 in a Caucasian.<sup>5,18</sup> A more accurate predictor of obesity in South Asians is waist circumference and the International Diabetes Federation has suggested that waist circumference thresholds should be reduced in South Asians compared to white Caucasian populations. Also, South Asian populations may have an increased genetic susceptibility to deposit intra-abdominal fat, particularly when moving to a sedentary and more affluent lifestyle, and some of this may be programmed in early foetal life.<sup>21</sup>

### Microvascular disease in South Asians

#### Retinopathy

Opinion is divided as to whether diabetic retinopathy is higher in South Asians. The United Kingdom Prospective Diabetes Study (UKPDS) showed similar prevalence of

retinopathy in South Asians compared to white Caucasians at their initial visit.<sup>9</sup> Conversely, the Southall study showed South Asians were 1.5 times more likely to have laser treatment for diabetic retinopathy,<sup>7</sup> and more recent studies showed retinopathy to be more prevalent in South Asians and also to appear at a younger age.<sup>22,23</sup> Many of these studies were carried out in a secondary care setting; therefore the true prevalence in the community is not known, and there is a real need for a large and definitive study of the prevalence and severity of diabetic retinopathy in South Asians. Overall to date, studies suggest that South Asians present with the microvascular complications of retinopathy at a younger age and earlier in the course of their type 2 disease and that this leads to poorer visual outcomes.<sup>7,9,22,23</sup>

#### Microalbuminuria and nephropathy

Microalbuminuria is both a marker for nephropathy and an independent predictor of cardiovascular and all-cause mortality in patients with type 2 diabetes. South Asians are more likely than white Caucasians to have microalbuminuria.<sup>24</sup> The relationship between BP and microalbuminuria in a recent study<sup>15</sup> confirmed that the prevalence of microalbuminuria was





significantly higher in South Asians compared with white Caucasians (31% vs 20%,  $p < 0.007$ ) and there is direct relationship between microalbuminuria and hypertension. Interestingly, in this study, among patients with normal untreated BP, the proportion that had microalbuminuria was three times higher in South Asians compared with white Caucasian groups (30.7% vs 10.1%,  $p < 0.049$ ). Figure 2 highlights the higher prevalence of microalbuminuria and proteinuria with increasing systolic BP in South Asians.

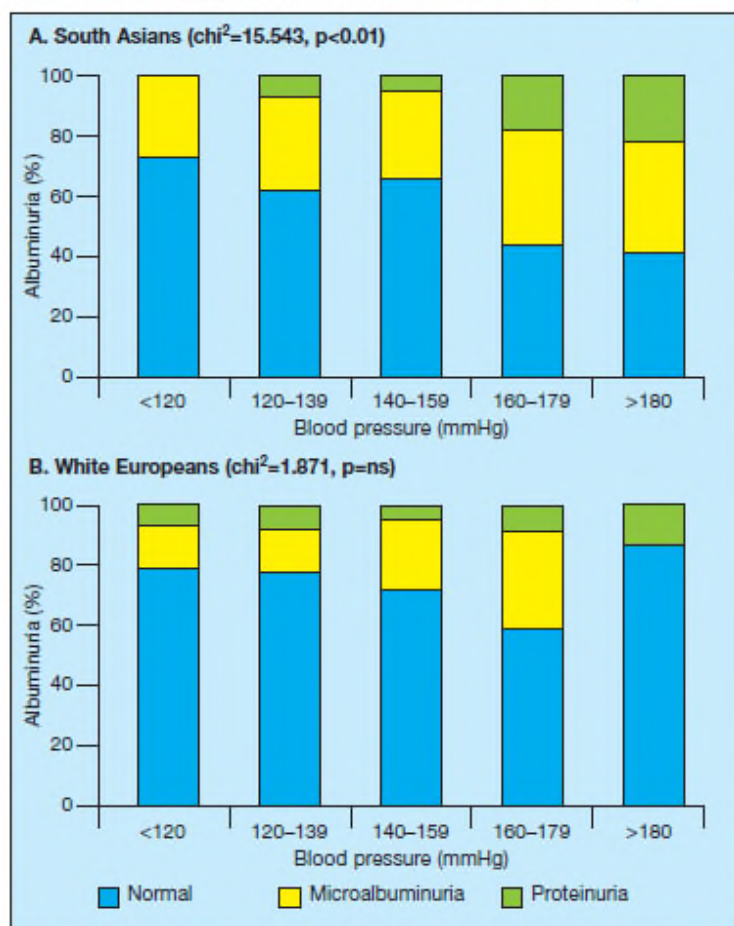
### Meeting the challenge

The South Asian community carries higher cardiovascular risk but is not adequately educated, screened and treated to reduce this risk. Finding and then aggressively treating the reversible risk factors – such as central obesity, reduced exercise, raised lipids, BP and diabetes – through raised awareness and effective screening remains a challenge.<sup>5</sup> Improving the awareness about the risks of CVD in the South Asian communities, and CVD's links to the metabolic syndrome and type 2 diabetes, is a vital step. Education to foster awareness of diabetes and all modifiable risk factors – such as obesity, sedentary life habits and smoking – needs to be improved. This population requires approaches that are community based and deliver health care which is sensitive to the needs of each ethnic group respecting their culture, customs, religion and lifestyle.<sup>5,25</sup>

Despite clear evidence of higher cardiovascular morbidity and mortality,<sup>10,11,13,14</sup> South Asians are less likely to be prescribed statins or sufficient anti-hypertensive drugs to reduce cholesterol and BP to internationally accepted targets in type 2 diabetes.<sup>5</sup>

The overall cardiovascular risk in the South Asian with diabetes is three-fold higher than that of a non-diabetic South Asian, and the risk is a further 50% higher when compared with a non-diabetic Caucasian. Despite these enormous risk differences, the major clinical trials of statins – e.g. CARDS,<sup>26</sup> HPS<sup>27</sup> and ASCOT-LLA<sup>28</sup> – do not provide sufficient or definitive evidence for South Asians. Current recommendations

**Figure 2.** Albuminuria status (%) and systolic blood pressure by ethnicity (chi<sup>2</sup> test for trend). (Reproduced by permission of © Sherborne Gibbs [Dixon AN, et al. Prevalence of microalbuminuria and hypertension in South Asians and White Europeans with Type 2 diabetes: A report from the United Kingdom Asian Diabetes Study (UKADS). *Diab Vasc Dis Res* 2006; 3: 22–25.]<sup>15</sup>)



for patients of South Asian origin are that virtually all type 2 diabetic patients will require a statin and that international guidelines of aiming for a total cholesterol level below 4 needs to be achieved. Non-diabetic South Asians need aggressive cholesterol lowering and, if conventional risk tables are used, many advocate adding a correction factor of 50%.<sup>5,29</sup>

Thresholds and targets for the treatment of cardiovascular risk factors are largely based on those derived from populations of Caucasians in the USA or North Europe. South Asians with a greater susceptibility to microvascular and

cardiovascular complications may need BP to be much lower. To achieve similar outcomes, South Asians with microalbuminuria need improved detection and effective action to achieve and maintain tighter BP targets. To achieve full protection, these targets may need to bring BP below 120/70mmHg.<sup>15</sup>

To reduce the increasing morbidity and mortality from diabetes and CVD in South Asians with type 2 diabetes, we need new approaches that take into account the specific needs of this community. The United Kingdom Asian Diabetes Study (UKADS)<sup>25</sup> was conceived following





**Table 1.** Risk factor change over one year of follow up in UKADS. (Reproduced by permission of © Blackwell Publishing [O'Hare JP, et al. Evaluation of delivery of enhanced diabetes care to patients of South Asian ethnicity: the United Kingdom Asian Diabetes Study (UKADS). *Diabetic Med* 2004; **21**: 1357–1365.])<sup>25</sup>

	Intervention mean difference	Control mean difference	Difference (95% CI)	p-value
Systolic blood pressure	-8.69	-2.11	-4.58 (-8.84 to -0.32)	0.035
Diastolic blood pressure	-3.14	+0.28	-3.41 (-5.66 to -1.16)	0.003
Total cholesterol	-0.51	-0.12	-0.38 (-0.65 to -0.12)	0.005
HbA <sub>1c</sub>	-0.23	-0.20	-0.03 (-0.36 to +0.30)	0.866

the publication of the UKPDS with the intention of developing models of health care for the South Asian communities that would improve risk factors.

### The United Kingdom Asian Diabetes Study

UKADS tested the idea that enhanced care for diabetes tailored to the needs of South Asian communities would improve risk factors for microvascular and cardiovascular complications ultimately reducing morbidity and mortality.<sup>25</sup> The pilot study published in 2004 included 361 patients of South Asian ethnicity with type 2 diabetes and risk factors and who were recruited from six general practices across Birmingham and Coventry (UK). Patients were randomised to either conventional or enhanced care.

Enhanced care included an additional practice nurse session per week supported by link worker sessions and a community diabetes specialist nurse. Patients in this group were followed up on average every two months in weekly clinics run by the practice nurses. Practice nurses worked with primary care physicians to implement the protocols and achieve targets for BP, lipids and glycaemic control. Each patient was contacted by a link worker before and between appointments to encourage clinic attendance. In addition, link workers provided interpretation and additional educational input to the patients to improve compliance and understanding, and to encourage dietary and lifestyle change. The community diabetes specialist nurse attended some of the research clinics and provided the practice teams with additional educational and clinical support, including experience in insulin initiation. Practices were actively encouraged to achieve tar-

gets and to adhere to treatment protocols. The group receiving conventional care were treated to the same protocols but did not receive any additional practice resources.

At the end of one year, it can be seen that there was a greater reduction in the mean systolic BP, diastolic BP and total cholesterol in the enhanced care group. There was no significant difference in the HbA<sub>1c</sub> between the groups (Table 1).

This pilot study showed clinically significant reductions in BP and total cholesterol in this high risk group. However, this needed to be confirmed in a large-scale study of a greater duration. UKADS 2, a follow-up study, is underway and due to complete later in 2007 to see whether this approach can provide sustained results in a larger population. The study has recruited approximately 1500 South Asian patients with type 2 diabetes from each of 18 practices in Birmingham and Coventry. Apart from them, 500 white Caucasian patients with diabetes have also been recruited as baseline controls. The study is designed to run for two years, recording cardiovascular risk markers and looking for major cardiovascular outcomes. In addition to that, a full health economic analysis will be performed to compare the effect of the Quality and Outcomes Framework initiative and whether the UKADS approach adds further value.

### Conclusion

South Asians in the UK seem to suffer excess morbidity and premature mortality due to an increased incidence and prevalence of type 2 diabetes with its enhanced cardiovascular risk. Much of this could be avoided. It is likely that this problem and the resulting health burden could increase due to the fact that

the second and third generation South Asians show similar characteristics and lifestyle habits to those of their parents and grandparents. As this population ages, the impact of increased microvascular and cardiovascular complications on local health authorities and care services will be greater. The areas with a high concentration of South Asians will thus need to find extra resources to meet future needs.

To reduce the morbidity and mortality from the complications of diabetes in South Asians, effective measures to control hypertension, dyslipidaemia and strict glycaemic control are urgently needed. Early identification of diabetes and the metabolic syndrome before complications ensue, with effective strategies for weight loss and enhanced physical activity, is clearly the cornerstone of a primary prevention programme. Further research needs to underpin the feasibility and costing for this approach.

For those patients with established diabetes, aggressive intervention targeting multiple risk factors with appropriate titration of statin doses, effective and multiple anti-hypertensive agents to target BPs below 130/80mmHg or lower if complications, low dose aspirin – together with a strict regimen to gain appropriate and achievable glycaemic control – is clearly the way forward, but often variably applied.

On a smaller scale, the UKADS pilot has demonstrated that an enhanced care package tailored to the needs of the South Asian community can in one year show significant improvements in BP and total cholesterol, and reduce overall cardiovascular risk. UKADS 2 on a larger scale, in a cluster randomised trial, is evaluating the same strategy





## Key points

- South Asians have a six-fold increase in their risk of developing type 2 diabetes
- The microvascular and cardiovascular complications of diabetes are more aggressive in South Asians
- Aggressive reduction of risk factors could reduce the excess morbidity and mortality seen in South Asians with type 2 diabetes
- Culturally sensitive community-based initiatives such as UKADS may lead to health care improvement for this group

with more aggressive targets to see if this approach is sustainable and cost effective.

## Acknowledgements

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## Conflict of interest statement

We would like to acknowledge the financial support for the UKADS project from Pfizer, Sanofi-Aventis, Servier, MSD/Schering Plough, Takeda, CSK, Bristol-Myers Squibb, Roche, El Lilly, Novo Nordisk, Merck, Boehringer Ingelheim and Daiichi Sankyo UK.

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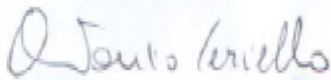
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Yours sincerely



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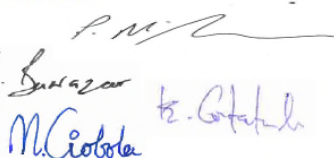
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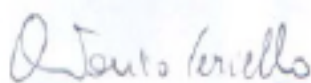
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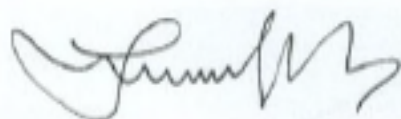
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If you require any further details please let me know.

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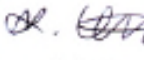



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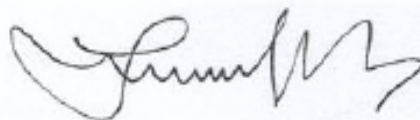
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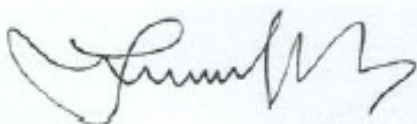
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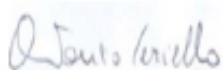
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6/4/16



*Metabolic endotoxaemia in childhood obesity*

**BMC Obesity (2016) 3:3DOI 10.1186/s40608-016-0083-7**

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*High Fat Intake leads to Acute Post-Prandial Exposure to Circulating Endotoxin in Type 2 diabetes mellitus Subjects*

**Diabetes Care 35: 375–382, 2012**

Alison L Harte (PhD)<sup>1</sup>, **Madhusudhan C Varma (MRCP)<sup>1</sup>**, Gyanendra Tripathi (PhD)<sup>1</sup>, Kirsty C McGee (PhD)<sup>1</sup>, Nasser M Al-Daghri (PhD)<sup>2</sup>, Omar S Al-Attas (PhD)<sup>2</sup>, Shaun Sabico (BSc)<sup>2</sup>, Joseph P O'Hare (MD)<sup>1</sup>, Antonio Ceriello (MD)<sup>3</sup>, Saravanan Ponnusamy (PhD)<sup>4</sup>, Sudhesh Kumar (MD)<sup>1</sup>, Philip G McTernan (PhD)<sup>1</sup>.

*Impact of acute hyperglycaemia on endothelial function and retinal vascular reactivity in patients with Type 2 diabetes*

**Diabetic Medicine 2011; 28: 4: 450-454**

**M. V. Chittari<sup>1</sup>**, P. McTernan<sup>2,3</sup>, N. Bawazeer<sup>2,3</sup>, K. Constantinides<sup>2,3</sup>, M. Ciotola<sup>4</sup>, J. P. O'Hare<sup>2,3</sup>, S. Kumar<sup>2,3</sup>, A. Ceriello<sup>5</sup>

*Increased Glycation and Oxidative Damage to Apolipoprotein B100 of LDL Cholesterol in Patients With Type 2 Diabetes and Effect of Metformin*

**DIABETES 2010; 59: 1038-1045**

Naila Rabbani, **Madhu Varma Chittari**, Charles W. Bodmer, Daniel Zehnder, Antonio Ceriello, and Paul J. Thornalley

## Invited Article

**M Varma Chittari**, K Bush, BM, S Bellary, S Kumar, AH Barnett, JP O' Hare

*Micro vascular and cardiovascular disease in South Asians: the emerging challenge*

Practical Diabetes International July / August 2007; 24: 328-332

## Abstract publications

*FGF21 action on human adipose tissue compromised by reduced  $\beta$ Klotho and FGFR1 expression in type 2 diabetes mellitus*

Milan K Piya<sup>1,2</sup>, Alison L Harte<sup>1</sup>, **Madhu V Chittari**<sup>1</sup>, Gyanendra Tripathi<sup>1</sup>, Sudhesh Kumar<sup>1,2</sup> & Philip G McTernan<sup>1</sup>

Endocrine Abstracts (2013) 31 P179 | DOI:10.1530/endoabs.31.P179

*Post-prandial high fat intake leads to acute exposure to circulating endotoxin in type 2 diabetes mellitus subjects*

Alison Harte<sup>1</sup>, **Madhusudhan Varma**<sup>1</sup>, Gyanendra Tripathi<sup>1</sup>, Kirsty McGee<sup>1</sup>, Nasser Al-Daghri<sup>2</sup>, Omar Al-Attas<sup>2</sup>, Shaun Sabico<sup>2</sup>, Joseph O'Hare<sup>1</sup>, Antonio Ceriello<sup>3</sup>, Ponnusamy Saravanan<sup>1,4</sup>, Sudhesh Kumar<sup>1</sup> & Philip McTernan<sup>1</sup>

Endocrine Abstracts (2012) 28 OC3.2

*Dynamic reactivity of micro-circulation is less pronounced in type 2 diabetic subjects than in BMI and aged matched controls following a glucose challenge: potential implications for future CVD.*

**M.S.V. Chittari Macharotu (Coventry /United Kingdom)**, P.G. McTernan, N. Bawazeer, K. Lois, P.J. O'Hare, M. Ciotola, S. Kumar, A. Ceriello.

European Heart Journal: 2009 ; 30 ( Abstract Supplement ), 358

Visfatin is Regulated By Rosiglitazone in Type 2 Diabetes Mellitus and Influenced by NFκB and JNK in Human Abdominal Subcutaneous Adipocytes

McGee KC, da Silva NF, Harte AL, Creely SJ, Hill MJ, Khanolkar M, Evans M, **Chittari MV**, Kumar S, McTernan PG.

Diabetologia: 2007: 50 supplement 1 page S58: 0126

Expression and Regulation by Insulin Sensitizer & TNFα of Visfatin in Human Adipose Tissue in Type 2 Diabetes

NF da Silva, KC McGee, AR Baker, AL Harte, SJ Creely, MJ Hill, M Khanolkar, **MV Chittari**, M Evans, S Kumar and PG McTernan

Diabetologia: September 2006: vol.49 supplement 1 page 423: 0697

Expression and Regulation by Insulin Sensitizer & TNFα of Visfatin in Human Adipose Tissue in Type 2 Diabetes

NF da Silva, KC McGee, AR Baker, AL Harte, SJ Creely, MJ Hill, M Khanolkar, **MV Chittari**, M Evans, S Kumar and PG McTernan

Diabetes: June 2006: Volume 55 Supplement 1 page A308: 1318-P

## **PRESENTATIONS AND POSTERS**

## **Presentations and Posters at Scientific meetings**

*High dose metformin therapy reduces glycation and oxidative damage to apolipoprotein B100 and may decelerate atherosclerosis in patients with type 2 diabetes*

N. Rabbani, **M. Varma Chittari**, D. Zehnder, A. Ceriello, P.J. Thornalley;  
Clinical Sciences Research Institute, University of Warwick, Coventry, UK.

45th Annual Meeting of the European Association for the Study of Diabetes (EASD) Vienna 29 September- 2 October 2009

### **Poster Presentation (1293)**

*Impact of the postprandial state on the risk of cardiovascular disease and retinopathy in type 2 diabetes.*

### **M Varma Chittari**

The 26<sup>th</sup> Annual Meeting of the Anglo Danish Dutch Diabetes Group ( ADDDG )  
Rutland, UK. 13<sup>th</sup> – 16<sup>th</sup> May 2008

#### **Oral presentation**

I presented my initial research results as a topic: Few of the slides in the presentation were short listed for the best slides at the end of the meeting.

*Visfatin is Regulated By Rosiglitazone in Type 2 Diabetes Mellitus and Influenced by NFκB and JNK in Human Abdominal Subcutaneous Adipocytes*

McGee KC, da Silva NF, Harte AL, Creely SJ, Hill MJ, Khanolkar M, Evans M, **Chittari MV**, Kumar S, McTernan PG

43rd Annual Meeting of the European Association for the Study of Diabetes (EASD)  
Amsterdam. 17-21 September 2007

#### **Oral Presentation**

*Expression and Regulation by Insulin Sensitizer & TNF $\alpha$  of Visfatin in Human Adipose Tissue in Type 2 Diabetes*

NF da Silva, KC McGee, AR Baker, AL Harte, SJ Creely, MJ Hill, M Khanolkar,  
**MV Chittari**, M Evans, S Kumar and PG McTernan

42nd Annual Meeting of the European Association for the Study of Diabetes ( EASD )  
Copenhagen-Malmoe, Denmark-Sweden. 14 - 17 September 2006

**Poster presentation**

*Expression and Regulation by Insulin Sensitizer & TNF $\alpha$  of Visfatin in Human Adipose Tissue in Type 2 Diabetes*

NF da Silva, KC McGee, AR Baker, AL Harte, SJ Creely, MJ Hill, M Khanolkar,  
**MV Chittari**, M Evans, S Kumar and PG McTernan

66th American Diabetes Association ( ADA ) Scientific Sessions  
Washington, D.C. 9 – 13 June, 2006

**poster presentation**

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